ISOLATION OF PHYTOPATHOGENIC FUNGI WITH BIOHERBICIDAL POTENTIAL AGAINST INVASIVE ALLIGATOR WEED, *Alternanthera philoxeroides*

Project report submitted to Sister Nivedita University in partial fulfilment of the requirement for the degree of

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IN

MICROBIOLOGY

Submitted By

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I hereby declare that the work which is being presented in this dissertation entitled "**Isolation of Phytopathogenic Fungi with Bioherbicidal Potential Against Invasive Alligator Weed, *Alternanthera philoxeroides***" in partial fulfilment of the requirements for the award of the degree of Master of Science in Microbiology is an authentic record of my own work carried out during the period **January, 2025** to **May, 2025** under the supervision of **Dr. Puja Ray**, Department of Life Sciences, Presidency University.

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CHAPTER 01

ABSTRACT

The spread of weeds such as Alligator weed (*Alternanthera philoxeroides*) has led to a rapid decrease in biodiversity within various aquatic ecosystems around the globe. These semi-aquatic species possess the capability to frequently outcompete indigenous species of an ecosystem, hence resulting in a loss of biodiversity. The extensive application of chemical herbicides has caused the rise of herbicide-resistant weed species, and a troubling decline in native species of plants and animals. This decrease is largely attributed to the enduring characteristics of the chemical herbicides, which can persist in the environment for extended periods. In light of these difficulties, it is crucial to develop non-toxic, environmentally friendly, affordable, and effective biological alternatives that can replace the chemical herbicides. Phytopathogenic fungi are disease-causing microorganisms that infect plants, resulting in various symptoms including wilting, blight, leaf discoloration, and decay. While they can negatively affect crop yields, their selective pathogenicity also makes them valuable for environmentally friendly weed management strategies. This study aimed to identify a suitable myco-herbicidal agent as an environmentally sustainable method for controlling the invasive weed. The study involved an approach beginning with various field surveys around Kolkata, West Bengal, for sample collection, followed by the isolation and screening of fungal isolates with potential herbicidal activity. The phytotoxic effect of crude metabolites of the isolates were assessed. Fifteen phytopathogenic fungi were isolated, among which, the fungi exhibiting the highest herbicidal effectiveness observed by disease severity on the weed underwent detailed morphological analysis. This effective isolate is identified as *Alternaria* sp. Although the fungus has been explored as a potential biocontrol agent for managing the weed in countries like Australia, Brazil, and China, extensive and long-term research is still essential to fully evaluate it and its bioactive compounds for development into effective bioherbicides targeting alligator weed.

CHAPTER 02

2.1. Keywords

Aquatic weed, Alligator weed, biocontrol, bioherbicide

2.2. Abbreviations

BOD - Biological oxygen demand; PDA - Potato dextrose agar; PDB - Potato dextrose broth; rpm – Rotation per minute: FD - Foliar damage

CHAPTER 03

INTRODUCTION

Alligator weed (*A. philoxeroides*) is a rapidly spreading and aggressive amphibious plant originating from the temperate zones of South America, primarily observed in waterways of countries like Brazil, Argentina and others (Julien et al., 1995; Holm et al., 1977). Initially restricted to the to the Paraná river belt in South America, it has now established itself to over 30 countries worldwide, including major regions such as the United States, India, China, Australia, Japan and several others (Zhu et al., 2017). Introduction of these invasive species to the non-native environments is believed to have occurred accidentally due to sediments that were trapped to the cargo of ships traveling from South America to various locations across the world during early 1990s.

(A) **(B)**

Fig.3.1. Alligator weed (*A. philoxeroides*) infested areas observed during field surveys **(A)** in aquatic environment; **(B)** in terrestrial environment

Alligator weed can be identified by its broad, oval-shaped leaves, small white flowers and a remarkable capacity to grow on both land as well as in water. Mature plants may have either single or branched stems, which are typically hollow. These stems can grow upright or lie flat, displaying a range of colours including light green, yellow, brown or red. The upright stems are usually dark green and can exceed more than 50 cm in height. There are a pair of leaves present at each node on the stem (Julien et al., 1995; Schooler et al., 2005). It is particularly challenging to control once established since it can spread by seeds, fragments and stalks. Because of its detrimental effects on local economy, biodiversity and water quality, it has been designated as a noxious weed in several places (Cilliers et al., 2003).

When conditions are favourable, the plant spreads out to create densely interconnected floating mats of stems which grows across the surface of the waterways. This thick plant mat has capabilities of blocking of the waterway and outcompeting the natives on the banks of the waterways (Buckingham, G. R. et al., 1996). These floating mats have the potential to be disturbed by water currents, especially during flooding events and they can limit water flow in creeks, canals, channels and drains hindering transport, boating and hinder irrigation and pumping systems, as well as disrupting the existence of native plant and animal species by hampering their natural habitat. (Holm et al., 1977; Julien et al., 1995).

(A) **(B)**

Fig.3.2. (A) White bloom of Alligator weed; **(B)** Broad, pointed and oval leaves of Alligator weed as observed during field survey

It has become a big issue in many regions of the world because of its invasive nature and rapid proliferation over in semi-aquatic and aquatic habitats. These mats contribute to increased sediment accumulation and create favourable conditions for mosquito breeding (Masoodi, Khan et al., 2012). These thick mats reduce water oxygen levels and make it more difficult to perform fishing activities (Schooler et al., 2005; Zhang et al., 2020). Under changing climatic conditions, the invasion potential of the weed is predicted to increase (Wu, Ismail, and Ding et al., 2017).

Alligator weed can host a variety of pests and diseases in addition to having an adverse effect on the environment. Researchers have looked into the possibility of employing biological control techniques to control its spread because of its potential to carry phytopathogens that can harm other plant species (Shen et al., 2005). Efforts to manage and restrict its spread include chemical treatments, mechanical

removal, and the use of biological control agents, but the plant's tenacity and capacity to regenerate from small fragments make it a persistent challenge. Alligator weed poses an extreme threat to waterways, wetlands and irrigated crops by blocking the waterways. It is not only capable of growing in aquatic environment but also on the terrestrial environment hence providing other native species a tough competition for growth and survival (Holm et al., 1977).

(A) **(B)**

Fig.3.3. (A) Alligator weed with disease symptoms observed during field survey; **(B)** diseased leaves of Alligator weed collected during field survey

Water bodies and surrounding land areas heavily infested with Alligator weed often become ideal breeding grounds for disease-carrying organisms, such as mosquitoes, snails and harmful microorganisms. This can bring out major health risks to human and animal communities residing nearby (Shen et al., 2005; Zhang et al., 2020).

Hence, there is a pressing need for environmentally friendly, cost-effective, and targeted biological solutions to manage invasive weed species. Certain plants, microorganisms, and fungi produce allelopathic compounds that can suppress weed growth. Among these, fungal pathogens and their metabolic byproducts have shown particular promise as biocontrol agents against alligator weed (Charudattan et al., 2001). Our study focuses on the control of alligator weed with its most promising fungal pathogen and the secondary metabolites released by the fungus.

CHAPTER 04

REVIEW OF LITERATURE

4.1. Invasiveness of Alligator weed, *Alternanthera philoxeroides*

A. philoxeroides is a vigorous invasive species that disrupts both aquatic and terrestrial ecosystems globally. It is a stoloniferous plant species that spreads primarily through vegetative propagation since its sexual reproduction is often limited or absent in invasive ranges. This asexual reproduction through stem fragments facilitates rapid colonization, especially in disturbed or moist environments (Julien et al., 1995). The plant creates dense mats across water surfaces, obstructing sunlight, lowering oxygen concentration in the water, and disrupting its natural flow. These effects can severely impact aquatic fauna and flora, resulting in altered ecosystem dynamics and biodiversity loss (Schooler et al., 2006). In terrestrial systems, it invades crop fields, pastures, and roadsides, thereby reducing agricultural productivity and increasing management costs.

One of the key features contributing to its invasiveness is its physiological durability. *Alternanthera philoxeroides* shows strong resilience to various environmental challenges, including low soil fertility, high salt concentrations and extreme temperature variations (Zhang et al., 2010). Its ability to shift between aquatic and terrestrial forms further complicates control efforts, making it one of the most challenging invasive weeds in tropical and subtropical regions.

4.2. Conventional Management Strategies

The management of *Alternanthera philoxeroides* has relied heavily on physical, chemical, and cultural methods. Mechanical removal, such as hand-pulling or cutting, is feasible for small infestations but is highly labour-intensive and often counterproductive if not meticulously conducted. Even small fragments left in the environment can regenerate, leading to renewed infestations (Julien & Stanley, 1999). Chemical herbicides, including glyphosate, metsulfuron methyl, diuron, dimethylamine, dichlobenil, fluridone and several others (Tanveer et al. 2018), have been used extensively in aquatic and terrestrial systems. However, concerns regarding the non-target effects, herbicide resistance, and environmental contamination have led to increasing scrutiny of these practices. The application of herbicides in water bodies is also tightly regulated due to potential impacts on aquatic life (Wilson et al., 2005). Cultural methods focus on ecosystem-based strategies such as nutrient management (to limit eutrophication and plant growth), competition from native species, and public education to prevent unintentional spread. However, these approaches often yield only marginal success when not integrated with more direct methods. Overall, the limitations of conventional control have prompted a shift toward more sustainable, ecologically sound alternatives such as biological control.

4.3. Biological Control Approaches

Biological control of *A. philoxeroides* relies on the intentional release of natural enemies, most often nematodes, insects, bacteria, and fungi or disease-causing organisms, from the native habitat of the weed to limit its spread and reproduction. It has shown considerable success in regions such as Australia, China, and certain areas of the United States (Julien & Griffiths, 1998).

Biological control has been investigated, and four host-specific insect species from South America have been released in several parts of the world against this weed (Julien and Griffiths et al., 1998). Of these, the flea beetle *Agasicles hygrophila* and the moth *Arcola malloi* were introduced into the USA and are exerting good control of floating mats of the weed, except in the extreme tropical and temperate areas (Julien and Griffiths et al 1998; Stewart et al., 2000).

The flea beetle *A. hygrophila* is regarded as the most effective traditional biological control agent used against the weed. It feeds on the leaf tissue, significantly reducing the weed's photosynthetic capacity. In regions with warm climates, such as southern China and northern Australia, this beetle has established well and dramatically reduced alligator weed infestations (Schooler et al., 2006). However, the efficacy of *A. hygrophila* is constrained by cold temperatures, making it less effective in temperate regions. To address this, complementary biocontrol agents such as the stem-boring moth *Arcola malloi* and the thrips *Amynothrips andersoni* have been introduced. These agents attack different parts of the plant and provide a more comprehensive suppression when used in combination.

There is growing interest in conservation biological control methods including strategies that involve the release of native or mass-reared agents or the conservation of natural enemies already present in the ecosystem. These strategies require careful monitoring to ensure environmental safety and long-term efficacy. Unlike its effectiveness in controlling floating mats, these insects have not controlled upland stands effectively. Hence, additional biocontrol agents are needed (Pomella, Robert & Raghavan et al., 2007).

4.4. Phytopathogenic Fungi as Bioherbicide

Phytopathogenic fungi have emerged as promising agents for developing mycoherbicides, biopesticides based on fungal pathogens specific to weed species. These bioherbicides are applied in much the same way as chemical herbicides but are typically more selective, posing fewer risks to non-target organisms and the environment (Boyetchko et al., 1996). Scientific activity in mycoherbicide studies has increased significantly and application of registered or unregistered mycoherbicides has also expanded worldwide (Hamid Cheraghian Radi, et al. 2020).

Fungi isolated from naturally infected *A. philoxeroides* populations often produce necrotic lesions, blights, or wilts that weaken or kill the plant. *Fusarium* sp., *Colletotrichum gloeosporioides*, *Alternaria* spp. and *Cercospora* spp. are among the most extensively investigated fungal pathogens. These fungi differ in their degree of host specificity and pathogenic intensity. For instance, *C. gloeosporioides* has shown significant leaf necrosis and stunting in controlled greenhouse trials, making it a promising candidate for bioherbicide development (Zhou et al., 2013).

Despite promising results, several challenges persist in commercializing fungal bioherbicides. These include the need for effective formulation techniques, issues with mass production, spore viability, and environmental stability. Ongoing research is enhancing the efficacy of these agents using surfactants, and synergistic formulations that improve infection rates and consistency under field conditions.

Research has shown promising results with several fungal agents. For instance, *Myrothecium roridum* has been tested against alligator weed and exhibited high pathogenicity, causing leaf blight and eventual plant mortality with minimal effects on non-target plants (Yang et al., 2005). These findings support its candidacy for further development as a selective mycoherbicide.

4.5. International Approaches

Several countries have developed programs to manage *A. philoxeroides* using integrated weed management (IWM). In Australia, biological control has formed the foundation of national management strategies, with *A. hygrophila* effectively reducing populations of the weed in aquatic habitats. Biological control has formed the foundation of national management strategies, with *A. hygrophila* effectively reducing populations of the weed in aquatic habitats. The agent has reduced management costs and minimized the use of chemical herbicides (Julien et al., 1995). Various fungal species have been explored for their biocontrol potential against alligator weed, with *A. alternantherae* and *Fusarium* spp. identified as promising candidates due to their observed pathogenicity (Evans, 1997).

In China, the weed has extensively spread throughout the southern and eastern regions of the country. A combination of biological agents (especially *A. hygrophila*) and localized herbicide application is employed (Ma et al., 2020). Research institutions have actively explored the potential of native phytopathogenic fungi). *A. alternantherae*, a native pathogen frequently associated with natural infections of alligator weed, and considered a strong contender for development into a bioherbicide (Zhou et al., 2004; Ma et al., 2020).

In Brazil, researchers have isolated several phytopathogenic fungi from infected alligator weed stands, including members of the *Cercospora* sp and *Colletotrichum* spp, which demonstrated potential for biological control in preliminary studies (Barreto & Evans, 1995).

The United States has implemented classical biological control and has mainly focused on insect biocontrol agents in the southeastern states. However, climatic limitations have restricted the success of *A. hygrophila* in northern regions. Therefore, integrated approaches, including manual removal and public awareness campaigns, are also promoted.

4.6. National Approaches (India)

In India, *A. philoxeroides* has emerged as a serious threat in several states, particularly in waterlogged and flood-prone regions. Conventional control measures such as herbicide application and manual removal are prevalent but often prove ineffective due to resource constraints and the weed's regenerative ability. Although still in the early stages, efforts to explore biological control—such as the introduction of *Agasicles hygrophila* have shown encouraging results in initial experimental trials (Patel et al., 2008).

Research institutions such as the Directorate of Weed Research (DWR) and various state agricultural universities have initiated studies on indigenous fungal pathogens as potential biocontrol agents. Multiple investigations in India have identified fungal pathogens naturally associated with diseased alligator weed. Pathogenic fungi such as *Fusarium* spp. and *Cercospora* spp. have been isolated and evaluated from diseased *A. philoxeroides* plants, identifying them as promising candidates for the creation of fungal-based bioherbicides (Kaur et al., 2015). Fungal strains such as *Curvularia*, and *Alternaria* also exhibited promising results. (Rao & Reddy, 2016; Prasad et al., 2019).

National policy has gradually shifted toward integrated weed management approaches, incorporating biological agents, public participation, and ecological restoration of infested habitats.

CHAPTER 05

OBJECTIVES

1. **Isolation of phytopathogenic fungus from infected Alligator weed leaves.**

2. **Pathogenicity Assessment of isolated fungus on healthy Alligator weed.**

3. **Production and extraction of secondary metabolites of fungal isolates.**

4. **Morphological identification of the most promising fungal strain with maximum herbicidal potential.**

CHAPTER 06

MATERIALS AND METHODS

6.1. Isolation of Fungus

6.1.1. Sample Collection

Field surveys were conducted in Kolkata city metropolitan area and its adjacent districts including North 24 Parganas, South 24 Parganas, Howrah and Birbhum, West Bengal, India (Table 1.1), from January 2025 to March, 2025, to mark alligator weed infested sites and collect alligator weed leaves with visible symptoms.

Survey Site	Latitude(°N)	Longitude (°E)	Laboratory Isolation Code
Dumdum, North 24 Parganas	22.619762	88.426483	ALI-4, ALI-15
Dumurjola Stadium, Howrah	22.582101	88.306739	ALI-2, ALI-3, ALI-9
Joka, South 24 Parganas	22.448200	88.301096	ALI-8
Kudghat, Kolkata	22.473288	88.344472	ALI-11, ALI-12, ALI-14, ALI-16
Kopai, Birbhum	23.712552	87.712555	ALI-5, ALI-6,ALI-7, ALI-10, ALI-13
New Alipur, Kolkata	22.508738	88.323594	No Isolates Obtained
Ramrampur, South 24 Parganas	22.272699	88.265591	No Isolates Obtained
Satragachi Jheel, Howrah	22.583195	88.294239	No Isolates Obtained

Table. 6.1. List of alligator weed-infested sites along with fungal isolates obtained from diseased leaves of alligator weed in districts of West Bengal, India from January 2025 to March 2025

Procedure

- Alligator weed leaves with visible disease symptoms accompanied by necrosis were collected from the alligator weed-infested sites.
- The collected samples were packaged in layers of dry tissue paper (Dutta and Ray et al., 2015).
- The specimens were transferred to plastic bags and maintained in a cold environment (Souza et al., 2017) during transportation to the laboratory.

6.1.2. Surface sterilization and cutting of the diseased portions of infected leaves

Surface sterilization was performed to remove the dust particles and microbial contaminants present on the surface of the leaves and the diseased portion of the leaves were separated.

Reagents Required

- Tap water
- Distilled water
- Ethanol (70%)

Materials Required

- Sterile petri plate
- Sterile scalpel
- Sterile pair of tweezers

Procedure

- Washed the infected leaves thoroughly in tap water to remove dirt and dust particles on the surface.
- Washed the surface of the leaves again in ethanol (70%) for 30 seconds.
- Rinsed the leaves with sterile distilled water (dH_2O) for 2 to 3 times (Dutta et al., 2021).
- The visible necrotic or chlorotic lesions on the leaves were kept in place on the sterile petri plate by a pair of tweezers and cut into small pieces with the help of a sterile scalpel (Basak, Dutta and Ray, 2024).

6.1.3. Preparation of Potato Dextrose Agar Media

Potato Dextrose Agar (PDA) is a solid general-purpose growth medium suitable for culturing fungi. It can be enhanced to suppress bacterial contamination. Additionally, it supports the growth of relevant phytopathogenic fungi. The nutrient-rich composition, derived from potato infusion, promotes sporulation and enhances pigment production in certain dermatophytes.

Reagents Required

- Distilled water
- Potato dextrose agar (PDA)
- Ethanol (70%)

Serial number	Constituents	Amount (g/1000ml)
1.	Potato infusion	200
2.	Dextrose	20
3.	Agar	15
4.	Distilled Water	1000

Table.6.2. Constituents of potato dextrose agar (PDA)

Materials Required

- Erlenmeyer's flask
- Sterile petri plates
- Sterile inoculation loop
- Spirit lamp
- Non-absorbent Cotton
- Autoclave
- Laminar air flow cabinet

Procedure

- Thoroughly cleaned all the glasswares and rinsed with ethanol (70%).
- Prepared desired amount of potato dextrose agar (PDA) media in an Erlenmeyer's flask utilizing the appropriate reagents (Table 6.2.) and evenly mixed them using magnetic stirrer.
- Autoclaved the media for 15 minutes at 121°C and a pressure of 15 psi to sterilize it.
- After media sterilization, pour in the sterile petri plates and leave until it cools down and solidifies with their lid partially open, inside the laminar air flow cabinet.

6.1.4. Inoculation of diseased leaves

Inoculation was the first step in isolation of fungal strains from infected parts of Alligator weed leaves. This involved proper placing of the infected parts onto solidified potato dextrose agar (PDA) media. The infecting fungus capable of growing on the media gave proper colonies. Each plate had maximum of four inoculated colonies.

Reagents Required

- Ethanol (99%)

Materials Required

- Sterile Inoculation loop
- Spirit lamp
- Autoclave
- Laminar air flow cabinet
- Non-absorbent cotton

Procedure

- Sterilized the inoculation loop by heating it till red hot, then immersed it in 99% ethanol to cool.
- Using the sterilized loop, carefully transferred small fragments of alligator weed leaf to the solid media.
- Re-sterilized the loop by reheating it till red hot, and cooled by gently waving it.
- Maximum of four pieces of leaves were inoculated in one petri plate containing PDA media.
- Covered the plates with parafilm properly, inverted the plates and incubated at 28°C for 3-5 days in a biological oxygen demand (BOD) incubator depending on the fungal growth.
- Stored the sealed incubated plates in the refrigerator al 4°C.

6.1.5. Subculturing to obtain pure culture

The process involved transfer of microorganisms from an old culture medium to a fresh medium with fresh supplied nutrients. This technique was employed to maintain the culture in an active state, prolong its viability, and increase the number of cells for various applications. In our experiment, this was repeated at 2 months interval to sustain the cells in active and healthy state. All pure cultures were stored at 4°C (Dutta et al., 2023).

Reagents Required

- Potato dextrose agar (PDA)
- Ethanol (99%)
- Sterile distilled water

Materials Required

- Sterile Inoculation loop
- Spirit lamp
- Non-absorbent Cotton
- Permanent marker pen
- Autoclave
- Laminar air flow cabinet

Procedure

- Cleaned all the glasswares thoroughly and rinsed with ethanol (70%).
- Prepared desired amount of potato dextrose agar (PDA) media in an Erlenmeyer's flask utilizing the appropriate reagents (Table 6.2.) and evenly mixed them using magnetic stirrer.
- Autoclaved the media for 15 minutes at 121°C and a pressure of 15 psi to sterilize it.
- After media sterilization, poured in the sterile petri plates and left with their lid partially open, until it cooled down and solidified, inside the laminar air flow cabinet.
- Properly labeled the solidified agar plates with the strain number.
- Sterilized the inoculation loop by heating it till red hot, then immersed it in 99% ethanol and allowed it to cool down.
- A part of fungal colony was cut with the help of the loop and placed it over the agar media (Basak, Dutta and Ray, 2024).
- Covered the plates with parafilm properly and incubated them at 27°C for 3-5 days in biological oxygen demand (BOD) incubator depending on the fungal growth.
- Stored the sealed incubated plates in the refrigerator al 4°C.

6.2. Twig Pathogenicity Test

Twig Assay was performed to evaluate the impact of isolated fungal phytopathogens on a plant by exposing twigs of the target weed to these agents under controlled conditions and check the effectiveness of fungal isolates on the growth of alligator weed twigs.

Insect and disease-free healthy plants of alligator weed were collected from areas and water bodies of different areas and grown under controlled conditions for 5 days in a conical flask filled with mineral water (Dutta et al., 2023). Twigs with 6-8 leaves were cut out and used in the twig pathogenicity test.

Reagents Required

- Tap water
- Distilled water
- Tween 20 solution (0.01%)

Materials Required

- Fungal isolates
- Twigs with 6-8 leaves
- Glass beaker
- Tissue paper
- Aluminum foil
- Absorbent Cotton
- Sterile scalpel
- Forceps
- Sterile Paint Brush
- Morter and Pestle

Procedure

- Cleaned glass beaker and wiped with ethanol (70%) and folded Tissue paper and put it in the beaker.
- Added few drops of tap water on the tissue to ensure a moist environment.
- Disease free twigs of alligator weed with 6-8 leaves were taken and surface sterilization of the twigs using ethanol (70%), and rinsed with distilled water (Basak, Dutta and Ray, 2024).
- Covered the bottom tip of the stalk with wet absorbent cotton and wrapped it with aluminum foil.
- Wiped a scalpel with ethanol (70%) and one square-centimeter of each isolated fungal colony was cut from the agar media plates (Basak, Dutta and Ray, 2024).
- Added a few drops of distilled water to the fungus and thoroughly mixed the solution using a mortar and pestle.
- With the help of a sterilized brush, applied the fungal mixture to the leaves and stem of Alligator weed twigs carefully and evenly (Basak, Dutta and Ray, 2024).
- With the help of a pair of forceps, kept the twigs inside the moisturized glass beakers and covered it with plastic wraps to ensure the moisture content.
- Repeated the similar procedure with Tween 20 solution (0.01%).
- Recorded observations on an interval of 1 day for 5 days (1st day, 3rd day and 5th day).

6.3. Whole Plant Bioassay

To evaluate bioherbicidal potential of phytopathogenic fungi, a whole plant bioassay was performed. This method involved using entire plant rather than just a part like leaves or twigs. Fungal isolates that caused mild or severe diseases in the twig assay, were taken. This method involved treating entire plant with fungal isolates to observe their effect on plant health and growth under controlled conditions. It allowed observation of disease symptom development like chlorosis, lesions or necrosis. By using this method, strongest herbicidal activity of different fungal isolates against target weed were screened.

Reagents Required

- Tap water
- Distilled water
- Tween 20 solution (0.01%)

Materials Required

- Healthy Alligator weed plants
- Isolated fungal colonies
- Erlenmeyer's flask
- Sterilized scalpel
- Sterilized forceps
- Sterilized paint brush
- Mortar and Pestle.

Procedure

- Cleaned the Erlenmeyer's flask and rinsed them with ethanol (70%).
- Disease free alligator weed whole plant with 8-12 leaves were taken and gently washed them with tap water
- The shoots were allowed to grow in the lab environment in Erlenmeyer's flasks supplemented with tap water for 5 days.
- Surface sterilization was carried out by washing the shoots with ethanol (70%) followed by distilled water (Basak, Dutta and Ray, 2024).
- Wiped a scalpel with ethanol (70%) and one square-centimeter of each isolated fungal colony was cut from the agar plates.
- Added few drops of Tween 20 solution (0.01%) to the cut-out colony and properly mix with the help of a mortar and pestle.
- Applied the fungal mixture to the leaves and stems of alligator weed with sterilized brush carefully.
- With the help of a pair of forceps, kept the whole plant inside the tap water filled Erlenmeyer's flasks and covered it with plastic wraps to ensure the moisture content.
- Recorded the presence or absence of disease by counting chlorosis (leaf yellowing) and necrosis on an interval of 1 day for 7 days (3^{rd} day, 5^{th} day and 7^{th} day).

6.4. Secondary metabolite extraction from isolated phytopathogenic fungus with toxic effects on alligator weed

6.4.1. Mass Culturing of the infecting fungus

Mass cultivation of fungi using Potato Dextrose Broth (PDB) is a widely employed technique in microbiological and biotechnological research. The liquid medium supports robust fungal growth and is especially suitable for producing large quantities of fungal biomass under controlled conditions. PDB offers an ideal nutrient environment composed of carbohydrates from dextrose and organic compounds from potato extract, promoting the proliferation of a wide range of fungal species. Large-scale fungal cultures prepared in Potato dextrose broth are essential for numerous applications, including the extraction of secondary metabolites, enzyme production, agricultural biocontrol studies, and taxonomic investigations. It involves scaling up the growth of a specific fungal strain from a small, isolated sample into larger quantities under controlled conditions.

Reagents Required

- Ethanol (70%)
- Distilled water
- Potato dextrose broth (PDB)

Serial Number	Constituents	Amount (g/1000ml)
1.	Potato infusion	200
2.	Dextrose	20
3.	Distilled water	1000

Table.6.3. Constituents of potato dextrose broth (PDB)

Materials Required

- Erlenmeyer's flask
- Non-absorbent cotton
- Sterile inoculation loop
- Spirit lamp
- Tissue paper
- Measuring cylinder

Procedure

- Cleaned Erlenmeyer's flasks properly and prepared potato dextrose broth (PDB) (as per Table 6.3.), uniformly mixed all the constituents with the help of a magnetic stirrer.
- Plugged the flasks with cotton and autoclaved at 121°C, 15 psi for 15 minutes.
- After sterilization, cooled the media to room temperature before inoculation of the fungal strains.
- Heated the inoculation loop until red hot, dipped it in ethanol (99%) to cool it and cut 1 square-centimetre of fungal colony and put it in the Erlenmeyer's flask containing the media (Basak, Dutta and Ray, 2024).
- Heated the mouth of flask and plugged it tightly with cotton before and after inoculation.
- Incubated them at 27°C for 21 days in a BOD incubator.

6.4.2. Extraction of crude metabolites

Fungi produce a wide variety of bioactive secondary metabolites with significant applications in agriculture. When cultured in nutrient-rich liquid media like Potato Dextrose Broth (PDB), many fungi secrete metabolites into the surrounding medium or retain them within their biomass.

The extraction of these crude fungal metabolites involved separating the fungal biomass from the culture broth to isolate the metabolites from either the liquid medium or the fungal tissue. The extracted crude compounds were further analysed or tested to check their bioherbicidal potential.

Materials Required

- Centrifuge
- Centrifuge tubes
- Glass funnel
- Whatman filter paper
- Erlenmeyer flask
- Paper label
- Permanent marker

Procedure

- Poured the media from the flasks containing freshly incubated fungus, into centrifuge tubes ensuring minimum cells into the centrifuge tubes.
- Centrifuged at 6,000 rpm for 15 minutes at 4°C to separate the biomass from culture (Basak, Dutta and Ray, 2024).
- Filtered the centrifuged supernatant with the help of Whatman filter paper into cleaned Erlenmeyer's flasks.
- Collect the filtrates in centrifuge tubes again and centrifuge at 6000 rpm for 15 minutes at 4°C.
- Filter the centrifuged supernatant again with Whatman filter paper into the Erlenmeyer flasks.
- Plug the flasks tightly with the help of cotton and store the filtrates at 4°C.

6.4.3. Toxicity Assessment of Fungal Metabolites

The use of fungal secondary metabolites as bioherbicidal agents presents a promising approach for the management of invasive weed species like alligator weed. Evaluating the phytotoxic effects of fungal metabolites on this weed helps in identifying potential natural compounds for weed control.

It was performed to assess the phytotoxic effects of crude fungal metabolites on alligator weed under controlled experimental conditions, aiming to explore their potential use as environmentally friendly herbicidal agents. Healthy, uniform alligator weed plants or cuttings were collected and grown in under controlled conditions to ensure acclimatization before treatment. This test assessed the potential harmful effects of metabolites of fungal isolates on the concerned weed.

Reagents Required

- Ethanol (70%)
- Distilled water

Materials Required

- Disease free alligator weed with 8-12 leaves.
- Tap water
- Glass beakers
- Plastic covers
- Spray bottles

Procedure

- Cleaned Erlenmeyer's flasks and wiped them with ethanol (70%).
- Disease free alligator weed whole plant with 8-12 leaves and gently washed them with tap water and grown in the lab environment in Erlenmeyer's flasks supplemented with tap water for 5 days.
- Surface sterilization was carried out by washing with ethanol (70%).
- Thoroughly cleaned the spray bottles using 70% ethanol followed by rinsing with distilled water, then filled it with the extracted metabolites from fungal isolates.
- Applied the metabolites by spraying into alligator weed whole plant until the leaves are completely saturated and covered the set up with plastics wraps to hold the moisture.
- Repeat the same at 5 days intervals (day 05 and day 10).
- Record observations on an interval of 5 days for 15 days (5^{th} day, 10^{th} day and 15^{th} day).
- Recorded the presence or absence of disease. The severity of infection was evaluated by counting chlorosis (leaf yellowing) and necrosis.

6.5. Statistical Analysis

Statistical Analysis was performed on effects exerted by fungal pathogens and their metabolites on alligator weed. Disease severity was determined by calculating the percentage of foliar damage:

[Percentage of foliar damage = (number of damaged leaves/ total number of leaves) × 100)]

Data from experiments were analysed using analysis of variance (ANOVA). This method determined whether there are significant differences between various groups in the study (Gomez et al., 1984). Once significant effects are identified through ANOVA, Post-hoc test (Bonferroni's test) are applied. These comparisons helped identify which fungal isolates or metabolite produced the most pronounced effects on the target weed (Zar et al., 1999).

All analyses were carried out using statistical software SPSS, Version 30.0.0.0 (172), which ensured precision. This statistical analysis is essential for understanding the effectiveness of fungal-based biocontrol agents and their effects.

6.6. Morphological Identification

Fungi associated with plant diseases present a wide range of morphological traits, making morphological analysis a tool for correct identification. This study aims to detect and define the morphological characteristics of fungal spores to facilitate taxonomy classification.

Fungal isolates obtained from symptomatic alligator weed leaves were examined using the slide cellophane technique (Butler and Mann, 1959). Spore-producing structures can be clearly observed in their native configuration using this technique. The isolates were characterized based on several morphological parameters, including colony growth patterns and the structure and abundance of spores. These features were carefully compared with descriptions available in standard mycological references (Gilman et al., 1959) (Barnett et al., 1960) to ensure accurate identification. This morphological approach provided insights into the diversity of fungal species associated with alligator weed and potential pathogenicity.

5.5.1. Initial Morphological Identification of isolated Fungal strains directly from the fungal plates

Reagents Required

- Lactophenol cotton blue stain

Materials required

- Grease free microscopic glass slides
- Cellophane tape
- Cover slip
- Tissue Paper
- Microscope

Procedure

- Incubated sealed pure culture plate of fungal isolates at 28 °C for 15 days.
- Clean glass slides are taken and a drop of Lactophenol Cotton Blue was placed.
- A small piece of cellophane tape was cut and lightly pressed its adhesive side onto the fungal colony.
- The tape was placed on the stain placed on the slide.
- Cover the slide with folded tissue paper and gently rub to remove excess stain and observe the slides under microscope at 40X.

5.5.2. Staining of the infecting fungus from infected leaves

Reagents Required

- Lactophenol cotton blue stain

Materials required

- Grease free microscopic glass slides
- Cellophane tape
- Cover slip
- Tissue Paper
- Microscope

Procedure

- Alligator weed plant with 8-10 leaves were grown in Erlenmeyer's flask.
- Wiped a scalpel with ethanol (70%) and cut one square-centimeter of each colony from the agar plates and finely mix with the help of a mortar and pestle with tween 20 solution.
- With the help of a cleaned brush, applied the fungal mixture to the plants of alligator weed carefully and grow them for 5-7 days.
- Infected (necrotic/chlorotic) leaf from the plant was taken, covered with a cover slip and observed it directly under microscope at 40X.
- Put a drop of Lactophenol cotton blue stain on the infected leaf, covered it with a cover slip and observe under microscope (40X).

CHAPTER 07

RESULTS AND DISCUSSIONS

7.1. Inoculation of diseased leaves

Result

The inoculated diseased leaves of Alligator weed in potato dextrose agar (PDA) media showed different fungal colonies on each plate, observed after 3-5 days of incubation at 27°C. Fungal colonies were obtained from areas Dumdum, North 24 Parganas, Dumurjola Stadium, Howrah, Joka, South 24 Parganas, Kudghat, Kolkata and Kopai, Birbhum.

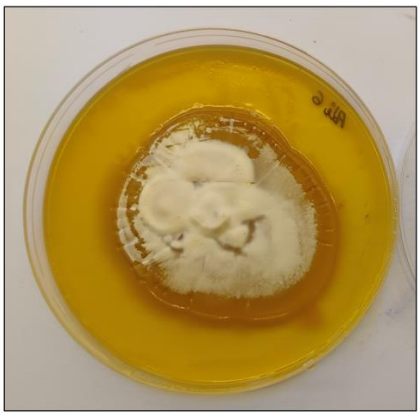
7.2. Pure cultures of isolated fungus

ALI-2

ALI-3

ALI-4

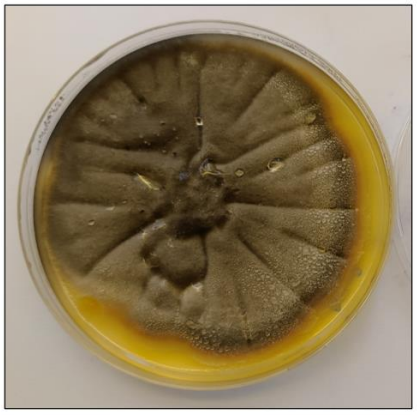
ALI-5



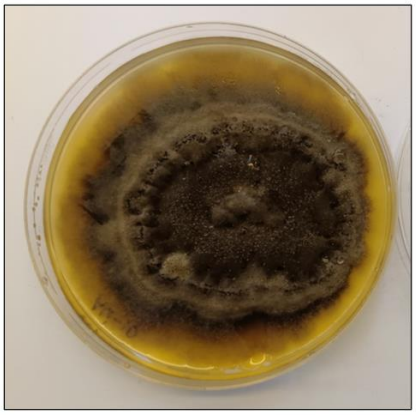
ALI-6

ALI-7

ALI-8



ALI-9



ALI-10

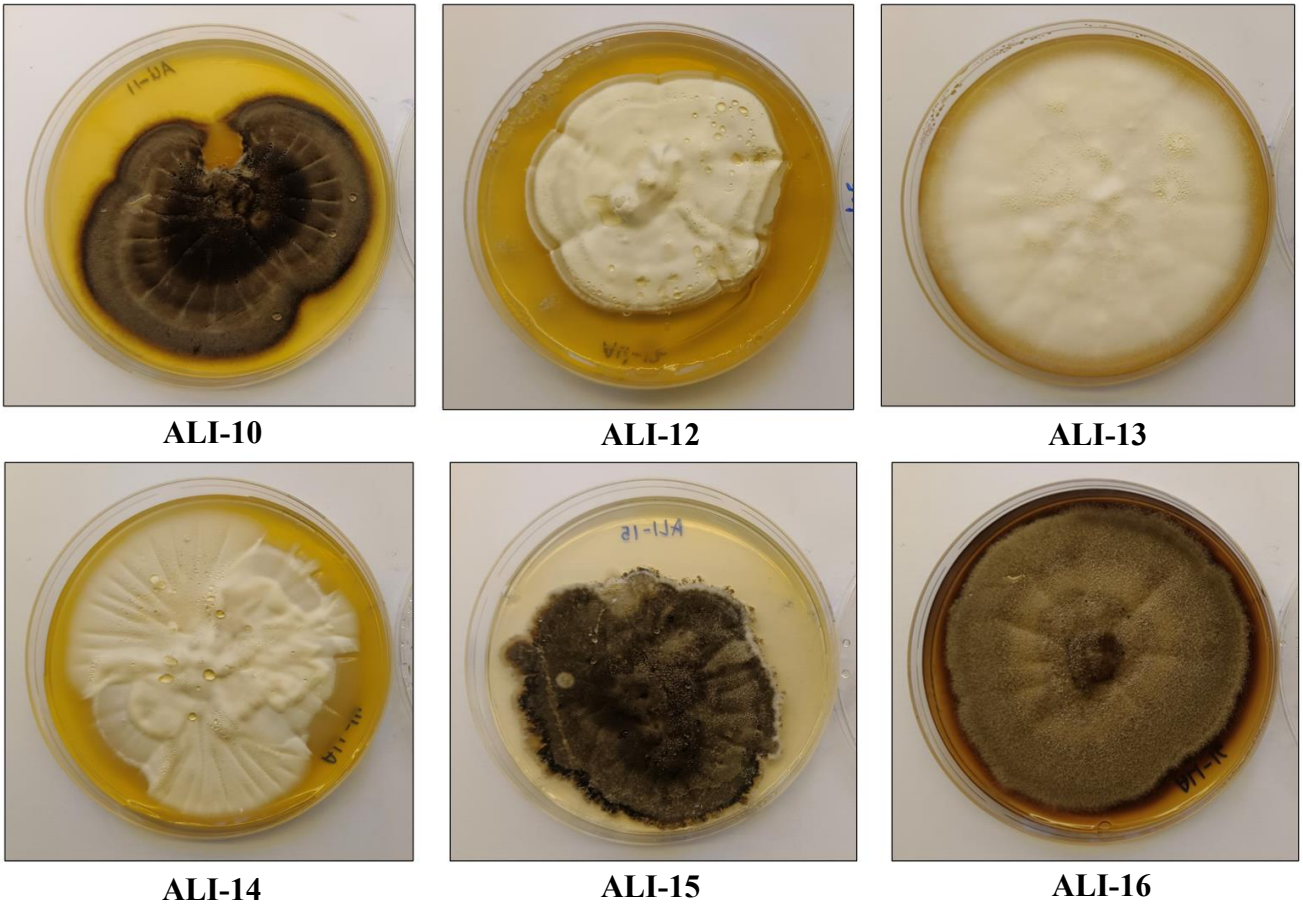


Fig.7.2.1. Pure culture plates of isolated fungus observed on PDA media

Result:

Total of **15** different fungal strains were isolated in pure culture and Laboratory Accession codes were added to all of the isolates.

Laboratory accession code of isolated fungus	**Location from where the diseased leaves were collected**
ALI – 2	Dumurjola Stadium, Howrah
ALI – 3	Dumurjola Stadium, Howrah
ALI – 4	Dumdum, North 24 Parganas
ALI – 5	Kopai, Birbhum
ALI – 6	Kopai, Birbhum
ALI - 7	Kopai, Birbhum
ALI – 8	Joka, South 24 Parganas
ALI – 9	Dumurjola Stadium, Howrah
ALI – 10	Kopai, Birbhum
ALI – 11	Kudghat, Kolkata
ALI – 12	Kudghat, Kolkata
ALI – 13	Kopai, Birbhum
ALI – 14	Kudghat, Kolkata
ALI – 15	Dumdum, North 24 Parganas
ALI - 16	Kudghat, Kolkata

Table 7.2.1. Isolated fungal pure cultures with laboratory accession codes and location from where they are isolated

7.3. Twig Assay

(1) Control:

(a) Healthy twig of Alligator weed + distilled water

Fig. 7.3.1. (a) Twig assay control with distilled water as observed on day 01, day 03 and day 05

(b) Healthy twig of Alligator weed + Tween 20 solution (0.01%)

Fig. 7.3.1. (b) Twig assay control with Tween 20 solution as observed on day 01, day 03 and day 05

(2) ALI-2

(a) Healthy twig of Alligator weed + ALI-2 fungus + distilled water

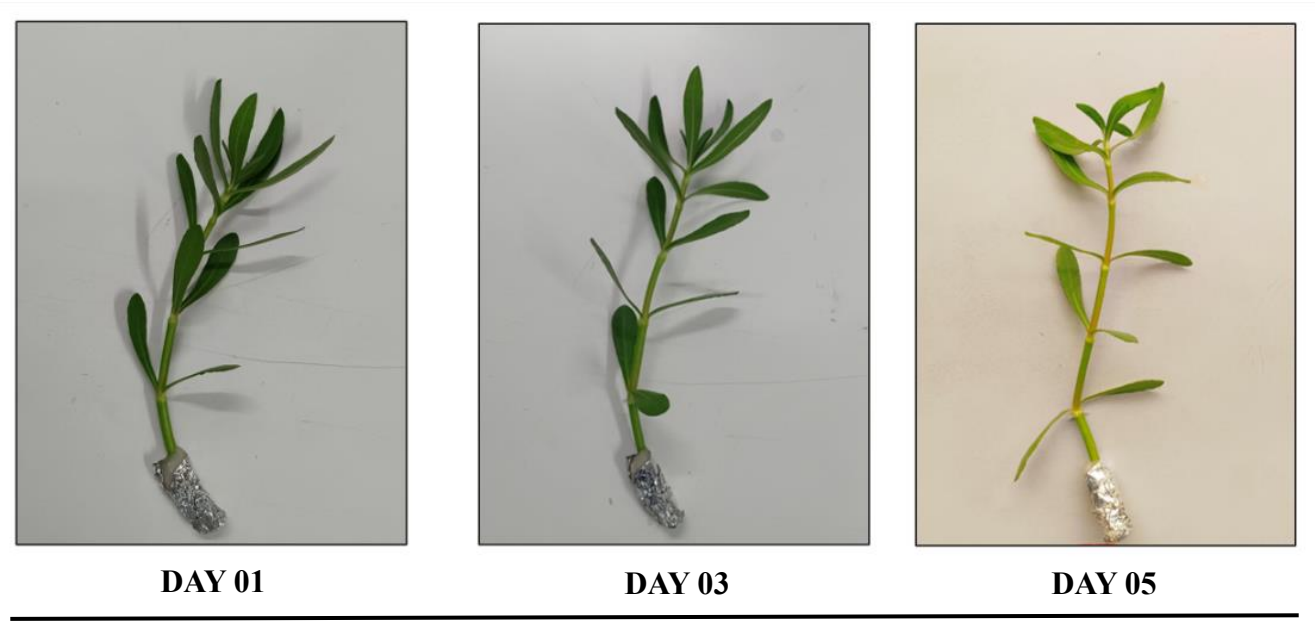


Fig. 7.3.2. (a) Twig assay of ALI-2 with distilled water as observed on day 01, day 03 and day 05

(b) Healthy twig of Alligator weed + ALI-2 fungus + Tween 20 solution (0.01%)

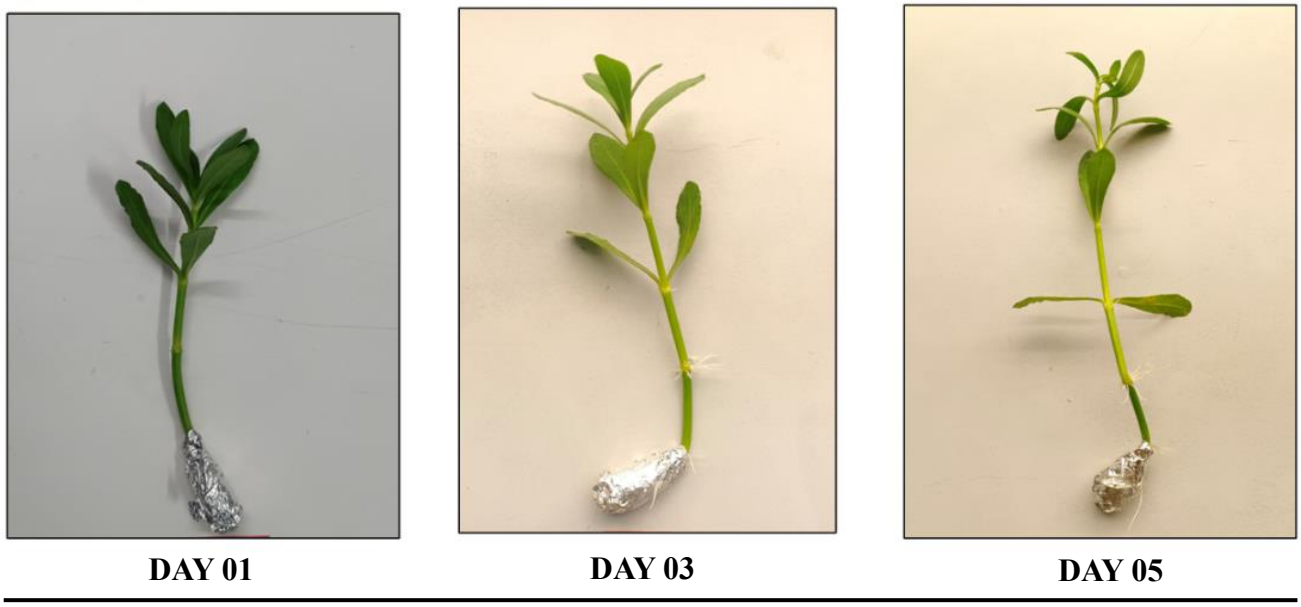


Fig. 7.3.2. (b) Twig assay of ALI-2 with Tween 20 solution as observed on day 01, day 03 and day 05

(3) ALI-3

(a) Healthy twig of Alligator weed + ALI-3 fungus + distilled water

Fig. 7.3.3. (a) Twig assay of ALI-3 with distilled water as observed on day 01, day 03 and day 05

(b) Healthy twig of Alligator weed + ALI-3 fungus + Tween 20 solution (0.01%)

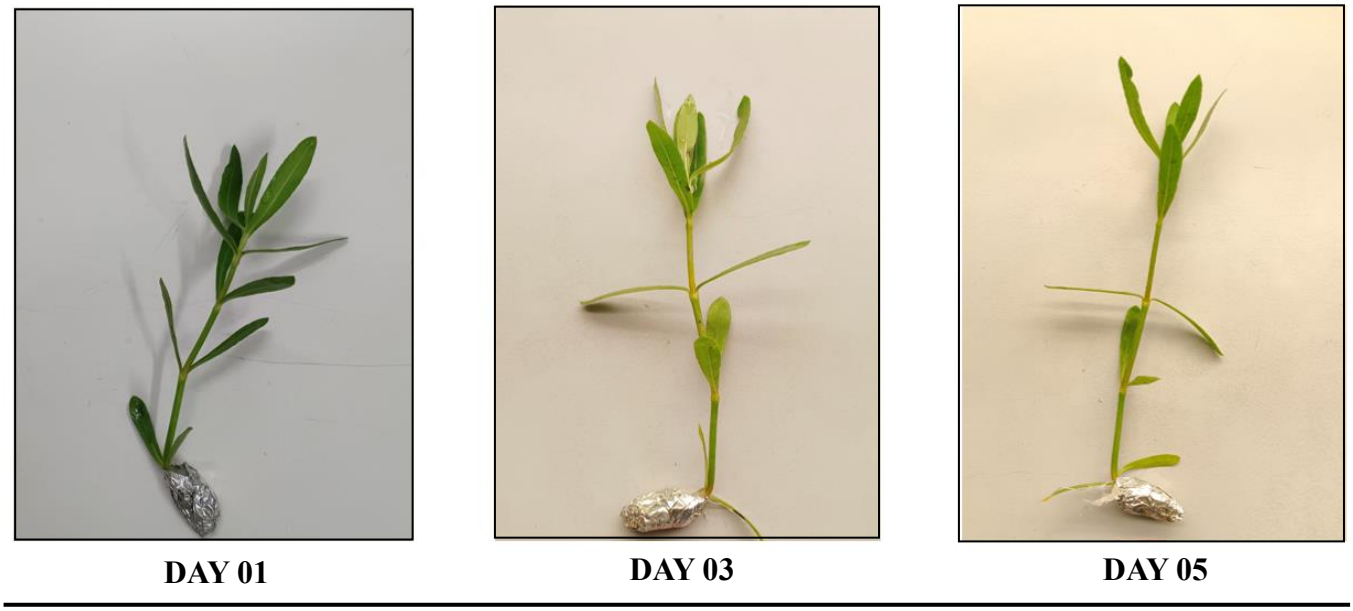


Fig. 7.3.3. (b) Twig assay of ALI-3 with Tween 20 solution as observed on day 01, day 03 and day 05

(4) ALI-4

(a) Healthy twig of Alligator weed + ALI-4 fungus + distilled water

Fig. 7.3.4. (a) Twig assay of ALI-4 with distilled water as observed on day 01, day 03 and day 05

(b) Healthy twig of Alligator weed + ALI-4 fungus + Tween 20 solution (0.01%)

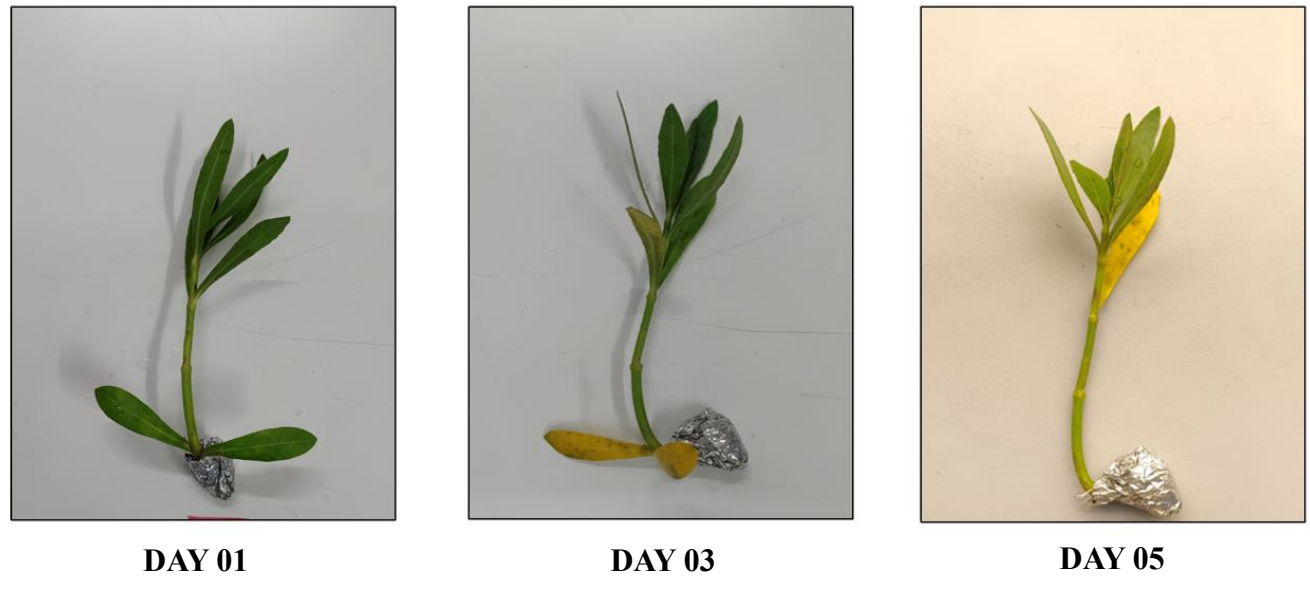


Fig. 7.3.4. (b) Twig assay of ALI-4 with Tween 20 solution as observed on day 01, day 03 and day 05

(5) ALI-5

(a) Healthy twig of Alligator weed + ALI-5 fungus + distilled water

Fig. 7.3.5. (a) Twig assay of ALI-5 with distilled water as observed on day 01, day 03 and day 05

(b) Healthy twig of Alligator weed + ALI-5 fungus + Tween 20 solution (0.01%)

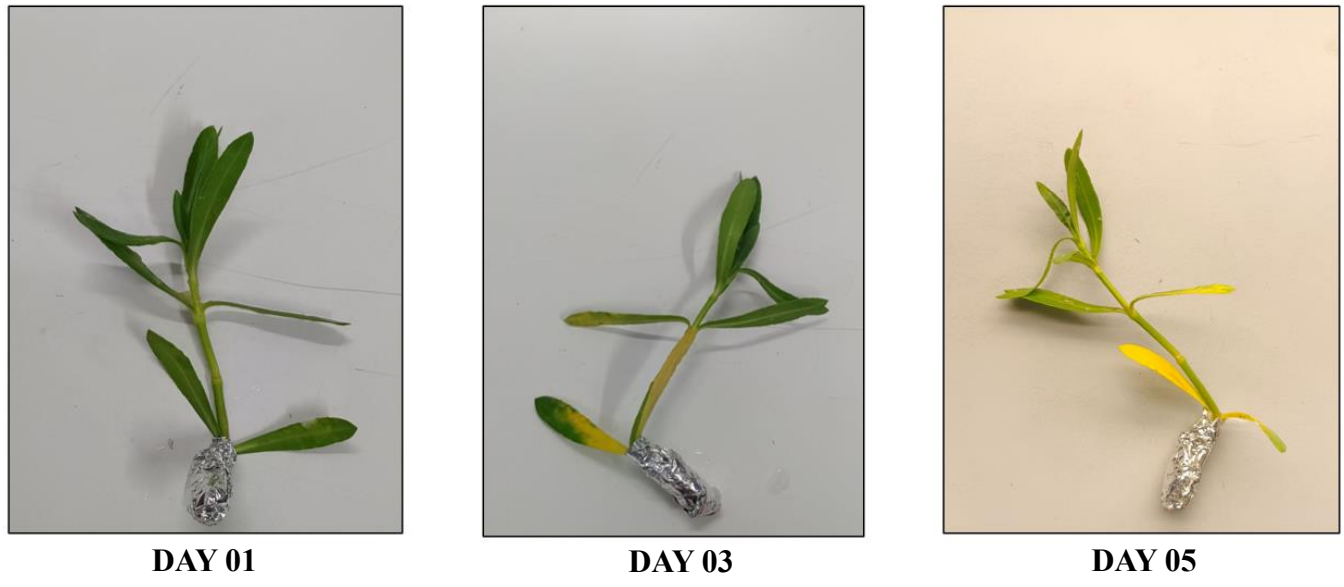


Fig. 7.3.5. (b) Twig assay of ALI-5 with Tween 20 solution as observed on day 01, day 03 and day 05

(6) ALI-6

(a) Healthy twig of Alligator weed + ALI-6 fungus + distilled water

Fig. 7.3.6. (a) Twig assay of ALI-6 with distilled water as observed on day 01, day 03 and day 05

(b) Healthy twig of Alligator weed + ALI-6 fungus + Tween 20 solution (0.01%)

Fig. 7.3.6. (b) Twig assay of ALI-6 with Tween 20 solution as observed on day 01, day 03 and day 05

(6) ALI-7

(a) Healthy twig of Alligator weed + ALI-7 fungus + distilled water

Fig. 7.3.7. (a) Twig assay of ALI-7 with distilled water as observed on day 01, day 03 and day 05

(b) Healthy twig of Alligator weed + ALI-7 fungus + Tween 20 solution (0.01%)

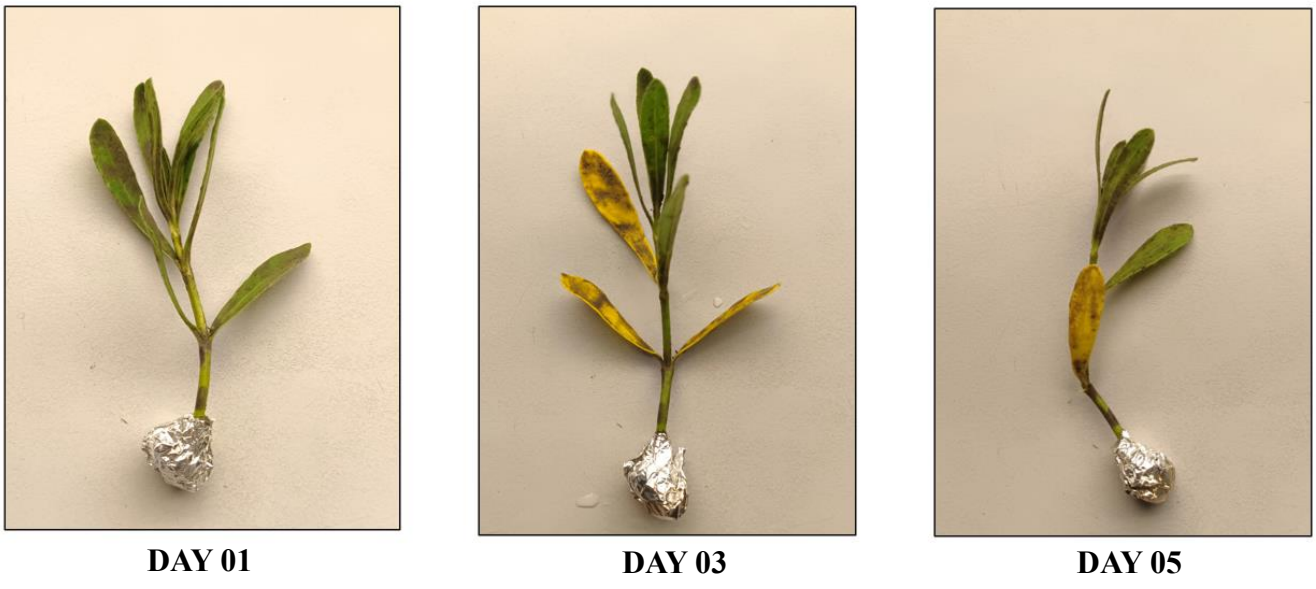


Fig. 7.3.7. (b) Twig assay of ALI-7 with Tween 20 solution as observed on day 01, day 03 and day 05

(8) ALI – 8

(a) Healthy twig of Alligator weed + ALI-8 fungus + distilled water

Fig. 7.3.8. (a) Twig assay of ALI-8 with distilled water as observed on day 01, day 03 and day 05

(b) Healthy twig of Alligator weed + ALI-8 fungus + Tween 20 solution (0.01%)

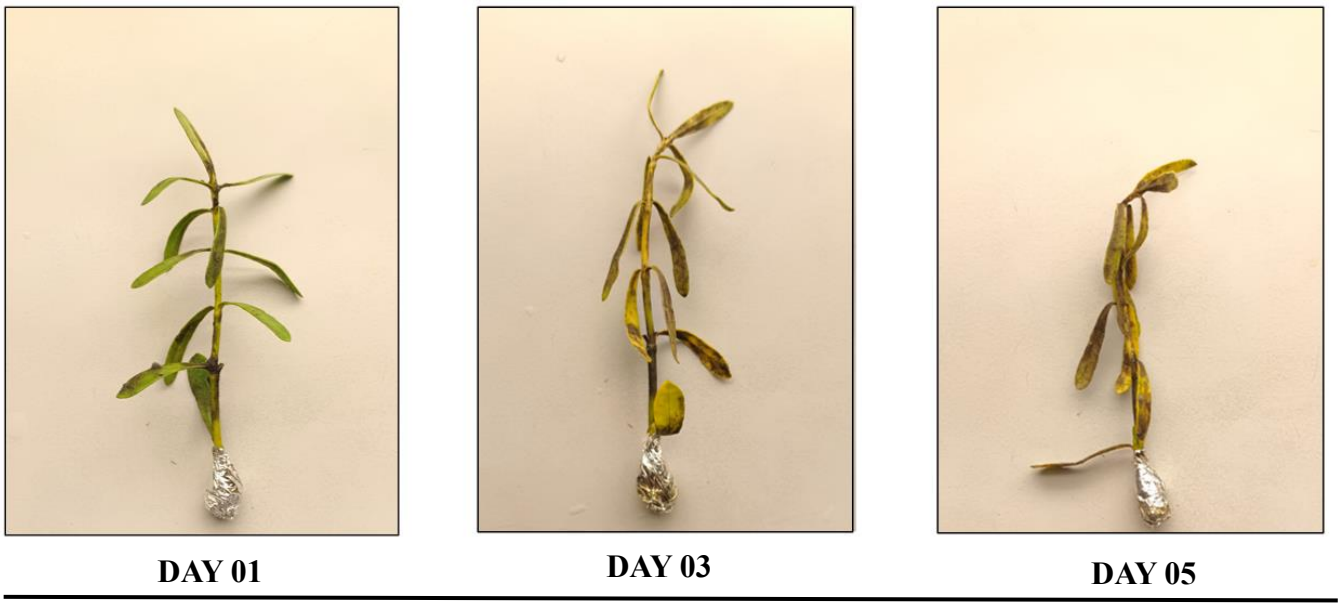


Fig. 7.3.8. (b) Twig assay of ALI-8 with Tween 20 solution as observed on day 01, day 03 and day 05

(9) ALI – 9

(a) Healthy twig of Alligator weed + ALI-9 fungus + distilled water

Fig. 7.3.9. (a) Twig assay of ALI-9 with distilled water as observed on day 01, day 03 and day 05

(b) Healthy twig of Alligator weed + ALI-9 fungus + Tween 20 solution (0.01%)

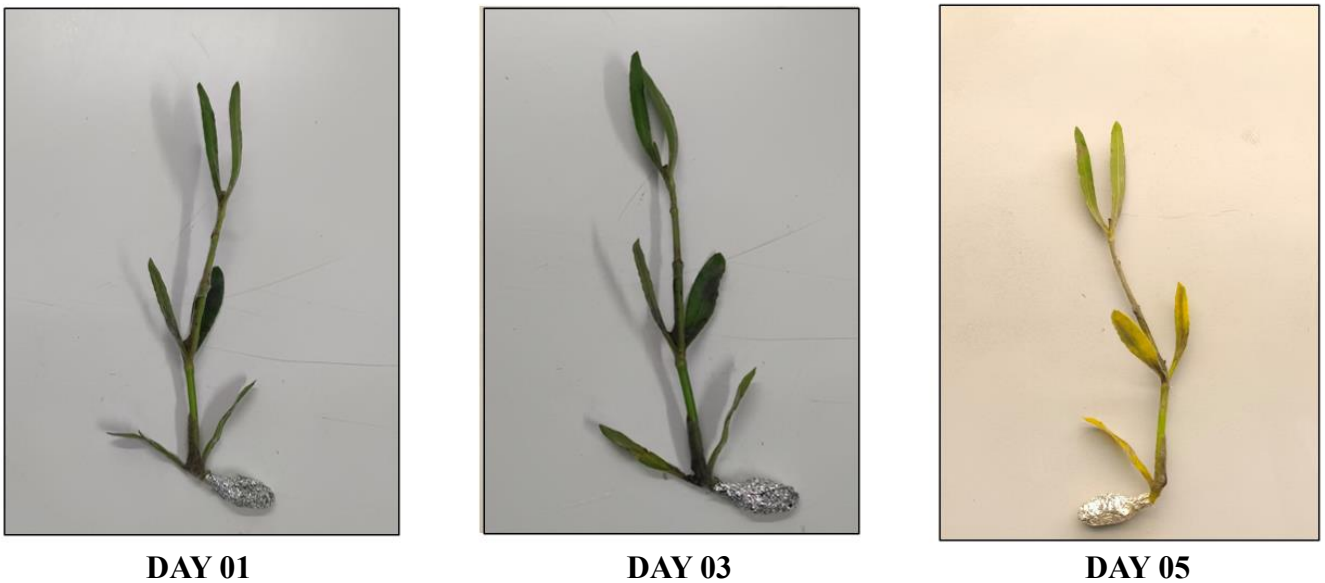


Fig. 7.3.9. (b) Twig assay of ALI-9 with Tween 20 solution as observed on day 01, day 03 and day 05

(10) ALI – 10

(a) Healthy twig of Alligator weed + ALI-10 fungus + distilled water

Fig. 7.3.10. (a) Twig assay of ALI-10 with distilled water as observed on day 01, day 03 and day 05

(b) Healthy twig of Alligator weed + ALI-10 fungus + Tween 20 solution (0.01%)

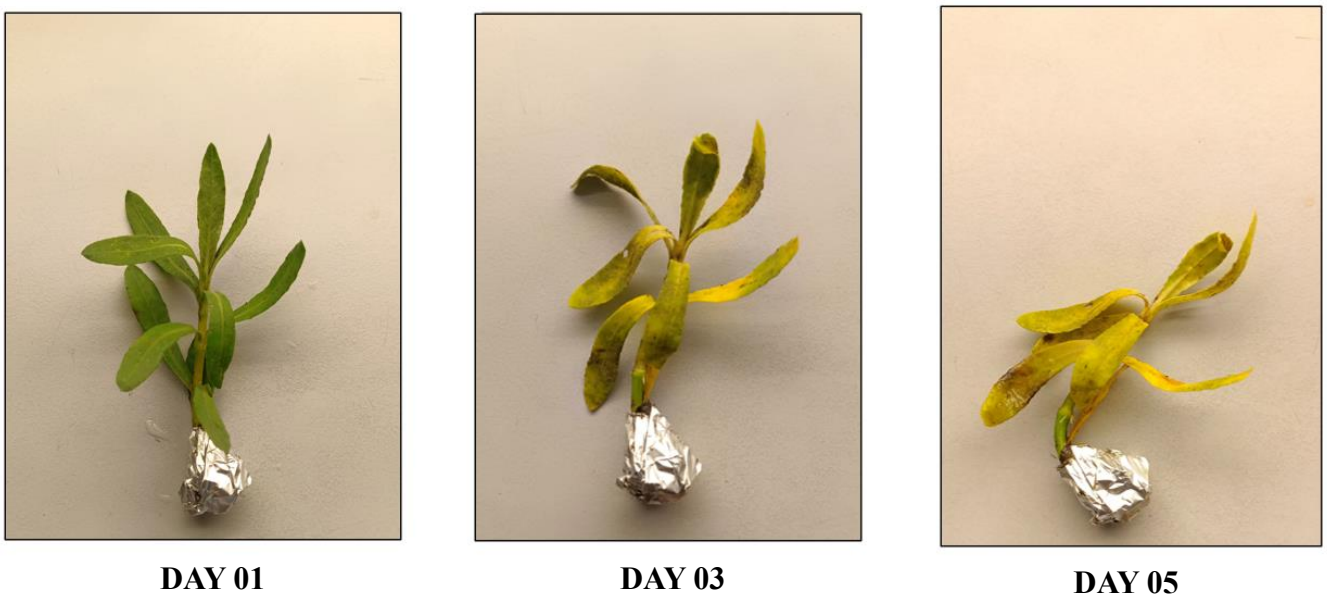


Fig. 7.3.10. (b) Twig assay of ALI-10 with Tween 20 solution as observed on day 01, day 03 and day 05

(11) ALI – 11

(a) Healthy twig of Alligator weed + ALI-11 fungus + distilled water

Fig. 7.3.11. (a) Twig assay of ALI-11 with distilled water as observed on day 01, day 03 and day 05

(b) Healthy twig of Alligator weed + ALI-11 fungus + Tween 20 solution (0.01%)

Fig. 7.3.11. (b) Twig assay of ALI-11 with Tween 20 solution as observed on day 01, day 03 and day 05

(12) ALI – 12

(a) Healthy twig of Alligator weed + ALI-12 fungus + distilled water

Fig. 7.3.12. (a) Twig assay of ALI-12 with distilled water as observed on day 01, day 03 and day 05

(b) Healthy twig of Alligator weed + ALI-12 fungus + Tween 20 solution (0.01%)

Fig. 7.3.12. (b) Twig assay of ALI-12 with Tween 20 solution as observed on day 01, day 03 and day 05

(13) ALI – 13

(a) Healthy twig of Alligator weed + ALI-13 fungus + distilled water

Fig. 7.3.13. (a) Twig assay of ALI-13 with distilled water as observed on day 01, day 03 and day 05

(b) Healthy twig of Alligator weed + ALI-13 fungus + Tween 20 solution (0.01%)

Fig. 7.3.13. (b) Twig assay of ALI-13 with Tween 20 solution as observed on day 01, day 03 and day 05

(14) ALI – 14

(a) Healthy twig of Alligator weed + ALI-14 fungus + distilled water

DAY 01 **DAY 03** **DAY 05**

Fig. 7.3.14. (a) Twig assay of ALI-14 with distilled water as observed on day 01, day 03 and day 05

b) Healthy twig of Alligator weed + ALI-14 fungus + Tween 20 solution (0.01%)

DAY 01 **DAY 03** **DAY 05**

Fig. 7.3.14. (b) Twig assay of ALI-14 with Tween 20 solution as observed on day 01, day 03 and day 05

(15) ALI – 15

(a) Healthy twig of Alligator weed + ALI-15 fungus + distilled water

Fig. 7.3.15. (a) Twig assay of ALI-15 with distilled water as observed on day 01, day 03 and day 05

b) Healthy twig of Alligator weed + ALI-15 fungus + Tween 20 solution (0.01%)

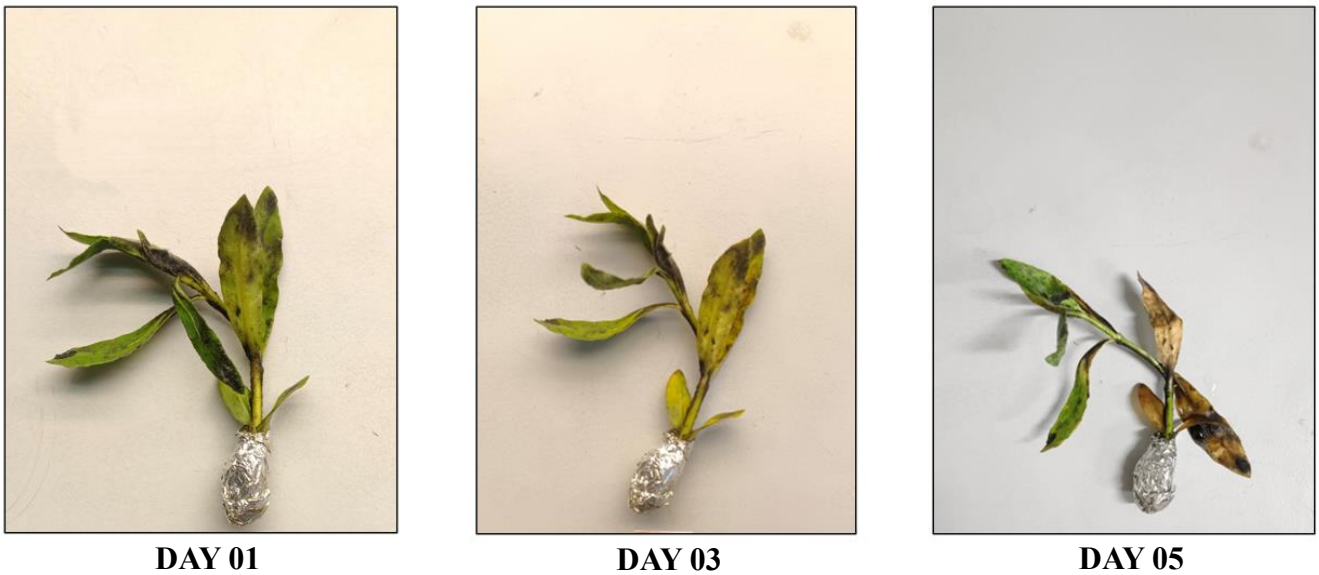
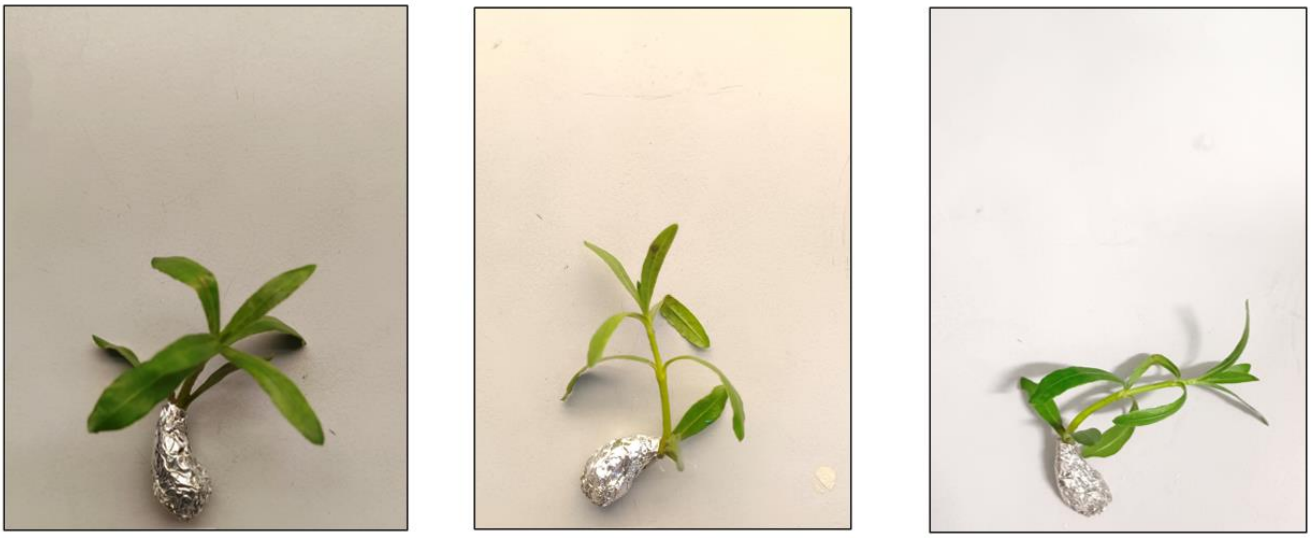


Fig. 7.3.15. (b) Twig assay of ALI-15 with Tween 20 solution as observed on day 01, day 03 and day 05

(16) ALI – 16

(a) Healthy twig of Alligator weed + ALI-16 fungus + distilled water



DAY 01 **DAY 03** **DAY 05**

Fig. 7.3.16. (a) Twig assay of ALI-16 with distilled water as observed on day 01, day 03 and day 05

b) Healthy twig of Alligator weed + ALI-16 fungus + Tween 20 solution (0.01%)

DAY 01 **DAY 03** **DAY 05**

Fig. 7.3.16. (b) Twig assay of ALI-16 with Tween 20 solution as observed on day 01, day 03 and day 05

Results:

Strain Number	Day 01	Day 03	Day 05
Control + dH_2O	No disease	No disease	No disease
Control + Tween 20	No disease	No disease	No disease
ALI - 2 + dH_2O	No disease	Mild disease	Mild disease
ALI - 2 + Tween 20	No disease	Mild disease	Severe disease
ALI - 3 + dH_2O	No disease	No disease	Mild disease
ALI - 3 + Tween 20	No disease	Mild disease	Severe disease
ALI - 4 + dH_2O	No disease	Mild disease	Severe disease
ALI - 4 + Tween 20	No disease	Mild disease	Severe disease
ALI - 5 + dH_2O	No disease	Mild disease	Mild disease
ALI - 5 + Tween 20	No disease	Mild disease	Severe disease
ALI - 6 + dH_2O	No disease	No disease	Mild disease
ALI - 6 + Tween 20	No disease	Mild disease	Severe disease
ALI - 7 + dH_2O	No disease	Mild disease	Mild disease
ALI - 7 + Tween 20	No disease	Mild disease	Severe disease
ALI - 8 + dH_2O	No disease	Mild disease	Mild disease
ALI - 8 + Tween 20	No disease	Severe disease	Severe disease
ALI - 9 + dH_2O	No disease	Mild disease	Severe disease
ALI - 9 + Tween 20	No disease	Mild disease	Severe disease
ALI - 10 + dH_2O	No disease	Mild disease	Severe disease
ALI - 10 + Tween 20	No disease	Severe disease	Severe disease
ALI - 11 + dH_2O	No disease	Mild disease	Mild disease
ALI - 11 + Tween 20	No disease	Mild disease	Severe disease
ALI - 12 + dH_2O	No disease	Mild disease	Severe disease
ALI - 12 + Tween 20	No disease	Mild disease	Severe disease
ALI - 13 + dH_2O	No disease	Mild disease	Mild disease
ALI - 13 + Tween 20	No disease	Mild disease	Mild disease
ALI - 14+ dH_2O	No disease	Mild disease	Mild disease
ALI - 14 + Tween 20	No disease	Mild disease	Severe disease
ALI - 15 + dH_2O	Mild disease	Severe disease	Severe disease
ALI - 15 + Tween 20	Mild disease	Severe disease	Severe disease
ALI - 16 + dH_2O	No disease	Mild disease	Mild disease
ALI - 16 + Tween 20	No disease	Mild disease	Mild disease

Table 7.3.1. Results of Twig Assay of different isolated fungal strains on healthy Alligator weed twigs

Discussions: Among the fifteen fungal isolates, all of the fifteen isolates caused leaf damage within 05 days of fungal inoculation. Tween 20 solution (0.01%) applied twigs demonstrated more damage as compared to distilled water applied twigs. No disease or damage was observed in the control.

All of the fifteen fungal isolates (ALI-2, ALI-3, ALI-4, ALI-5, ALI-6, ALI-7, ALI-8, ALI-9, ALI-10, ALLI-11, ALI-12, ALI-13, ALI-14, ALI-15 and ALI-16) showing results in twig assay were assessed by Whole plant Bioassay.

7.4. Whole Plany Bioassay

(1) Control:

Healthy whole plant of Alligator weed + Tween 20 solution (0.01%)

DAY 03 **DAY 05** **DAY 07**

Fig. 7.4.1. Whole plant Bioassay Control with Tween 20 solution as observed on day 03, day 05 and day 07 after inoculation

(2) ALI-2

Healthy whole plant of Alligator weed + ALI-2 fungus + Tween 20 solution (0.01%)

DAY 03 **DAY 05** **DAY 07**

Fig. 7.4.2. Whole plant Bioassay of ALI-2 with Tween 20 solution as observed on day 03, day 05 and day 07 after inoculation

(3) ALI-3

Healthy whole plant of Alligator weed + ALI-3 fungus + Tween 20 solution (0.01%)

DAY 03 **DAY 05** **DAY 07**

Fig. 7.4.3. Whole plant Bioassay of ALI-3 with Tween 20 solution as observed on day 03, day 05 and day 07 after inoculation

(4) ALI-4

Healthy whole plant of Alligator weed + ALI-4 fungus + Tween 20 solution (0.01%)

DAY 03 **DAY 05** **DAY 07**

Fig. 7.4.4. Whole plant Bioassay of ALI-4 with Tween 20 solution as observed on day 03, day 05 and day 07 after inoculation

(5) ALI-5

Healthy whole plant of Alligator weed + ALI-5 fungus + Tween 20 solution (0.01%)

DAY 03 **DAY 05** **DAY 07**

Fig. 7.4.5. Whole plant Bioassay of ALI-5 with Tween 20 solution as observed on day 03, day 05 and day 07 after inoculation

(6) ALI-6

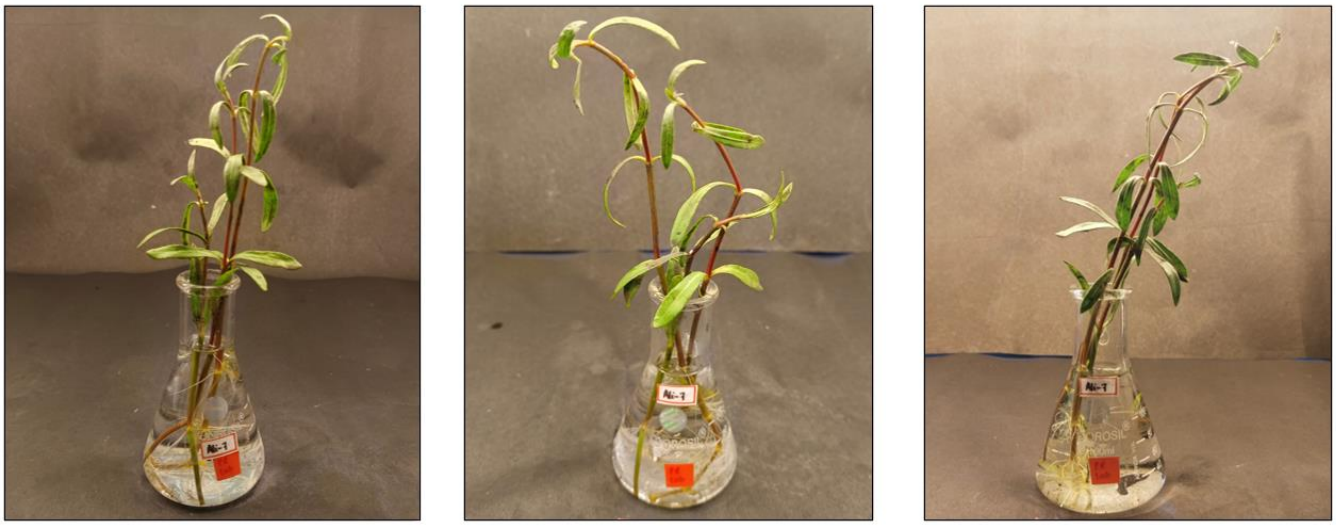
Healthy whole plant of Alligator weed + ALI-6 fungus + Tween 20 solution (0.01%)

DAY 07 **DAY 05** **DAY 07**

Fig. 7.4.6. Whole plant Bioassay of ALI-6 with Tween 20 solution as observed on day 03, day 05 and day 07 after inoculation

(7) ALI-7

Healthy whole plant of Alligator weed + ALI-7 fungus + Tween 20 solution (0.01%)



DAY 03 **DAY 05** **DAY 07**

Fig. 7.4.7. Whole plant Bioassay of ALI-7 with Tween 20 solution as observed on day 03, day 05 and day 07 after inoculation

(8) ALI-8

Healthy whole plant of Alligator weed + ALI-8 fungus + Tween 20 solution (0.01%)

DAY 03 **DAY 05** **DAY 07**

Fig. 7.4.7. Whole plant Bioassay of ALI-8 with Tween 20 solution as observed on day 03, day 05 and day 07 after inoculation

(9) ALI-9

Healthy whole plant of Alligator weed + ALI-9 fungus + Tween 20 solution (0.01%)

DAY 03 **DAY 05** **DAY 07**

Fig. 7.4.9. Whole plant Bioassay of ALI-9 with Tween 20 solution as observed on day 03, day 05 and day 07 after inoculation

(10) ALI-10

Healthy whole plant of Alligator weed + ALI-10 fungus + Tween 20 solution (0.01%)

DAY 03 **DAY 05** **DAY 07**

Fig. 7.4.10. Whole plant Bioassay of ALI-10 with Tween 20 solution as observed on day 03, day 05 and day 07 after inoculation

(11) ALI-11

Healthy whole plant of Alligator weed + ALI-11 fungus + Tween 20 solution (0.01%)

DAY 03 **DAY 05** **DAY 07**

Fig. 7.4.11. Whole plant Bioassay of ALI-11 with Tween 20 solution as observed on day 03, day 05 and day 07 after inoculation

(12) ALI-12

Healthy whole plant of Alligator weed + ALI-10 fungus + Tween 20 solution (0.01%)

DAY 03 **DAY 05** **DAY 07**

Fig. 7.4.12. Whole plant Bioassay of ALI-12 with Tween 20 solution as observed on day 03, day 05 and day 07 after inoculation

(13) ALI-13

Healthy whole plant of Alligator weed + ALI-13 fungus + Tween 20 solution (0.01%)

DAY 03 **DAY 05** **DAY 07**

Fig. 7.4.13. Whole plant Bioassay of ALI-13 with Tween 20 solution as observed on day 03, day 05 and day 07 after inoculation

(14) ALI-14

Healthy whole plant of Alligator weed + ALI-14 fungus + Tween 20 solution (0.01%)

DAY 03 **DAY 05** **DAY 07**

Fig. 7.4.14. Whole plant Bioassay of ALI-14 with Tween 20 solution as observed on day 03, day 05 and day 07 after inoculation

(13) ALI-15

Healthy whole plant of Alligator weed + ALI-15 fungus + Tween 20 solution (0.01%)

DAY 03 **DAY 05** **DAY 07**

Fig. 7.4.15. Whole plant Bioassay of ALI-15 with Tween 20 solution as observed on day 03, day 05 and day 07 after inoculation

(16) ALI-16

Healthy whole plant of Alligator weed + ALI-16 fungus + Tween 20 solution (0.01%)

DAY 03 **DAY 05** **DAY 07**

Fig. 7.4.16. Whole plant Bioassay of ALI-16 with Tween 20 solution as observed on day 03, day 05 and day 07 after inoculation

Results:

	DAY 03	DAY 05	DAY 07
Fungal Isolates	**FD(%) Mean ± SE**	**FD(%) Mean ± SE**	**FD(%) Mean ± SE**
ALI-2	8.33 ± 4.81	25 ± 4.81	52.77 ± 2.77
ALI-3	3.33 ± 3.33	10 ± 5.77	13.33 ± 8.81
ALI-4	6.66 ± 3.33	10 ± 5.77	50 ± 5.77
ALI-5	10 ± 5.77	16.66 ± 73.33	23.33 ± 6.66
ALI-6	7.5 ± 3.81	29.16 ± 15.02	32.5 ± 11.81
ALI-7	0 ± 0	0 ± 0	4.16 ± 4.16
ALI-8	0 ± 0	10 ± 10	40 ± 5.77
ALI-9	6.66 ± 3.33	19.16 ± 9.16	35 ± 20.20
ALI-10	9.44 ± 5.80	31.11 ± 5.87	53.33 ± 3.33
ALI-11	10 ± 5.77	16.11 ± 3.88	22.22 ± 4.00
ALI-12	6.66 ± 3.33	16.66 ± 6.66	20 ± 5.77
ALI-13	13.33 ± 6.66	27.5 ± 11.45	28.33 ± 1.66
ALI-14	7.14 ± 4.12	16.66 ± 13.25	52.38 ± 6.29
ALI-15	43.33 ± 6.66	56.66 ± 12.01	74.16 ± 8.20
ALI-16	6.66 ± 6.66	6.66 ± 6.66	25.83 ± 7.94
Control	0 ± 0	0 ± 0	0 ± 0

Table.7.4.1. FD(%) Mean of Whole plant bioassay of fungal isolates after 03, 05 and 07 days of inoculation

ANOVA Table (A)

			Sum of Squares	df	Mean Square	F	Sig.
FD (%) * STRAINS	Between Groups	(Combined)	4262.015	14	304.430	4.573	<.001
	Within Groups		1996.948	30	66.565		
	Total		6258.963	44			

ANOVA Table (B)

			Sum of Squares	df	Mean Square	F	Sig.
FD (%) * STRAIN	Between Groups	(Combined)	7654.383	14	546.742	2.484	.018
	Within Groups		6603.959	30	220.132		
	Total		14258.342	44			

ANOVA Table(C)

			Sum of Squares	df	Mean Square	F	Sig.
FD (%) * STRAINS	Between Groups	(Combined)	14451.455	14	1032.247	5.185	<.001
	Within Groups		5972.354	30	199.078		
	Total		20423.810	44			

Table.7.4.2. ANOVA Table for Whole plant bioassay of fungal isolates after **(A)** 03, **(B)** 05 and **(C)** 07 days, respectively

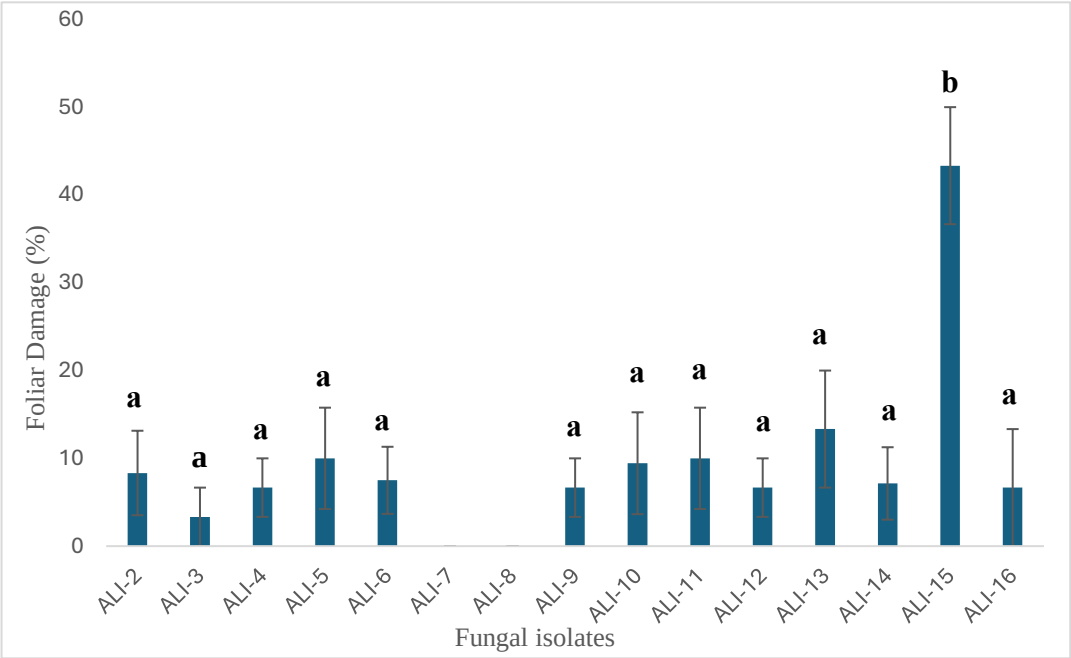


Fig.7.4.17. Effect of different fungal isolates on Alligator weed after 03 days of inoculation. Vertical bars represent the standard error of the mean. Means marked with the same letter(s) above the bars are not significantly different according to Bonferroni's test ($p < 0.05$).

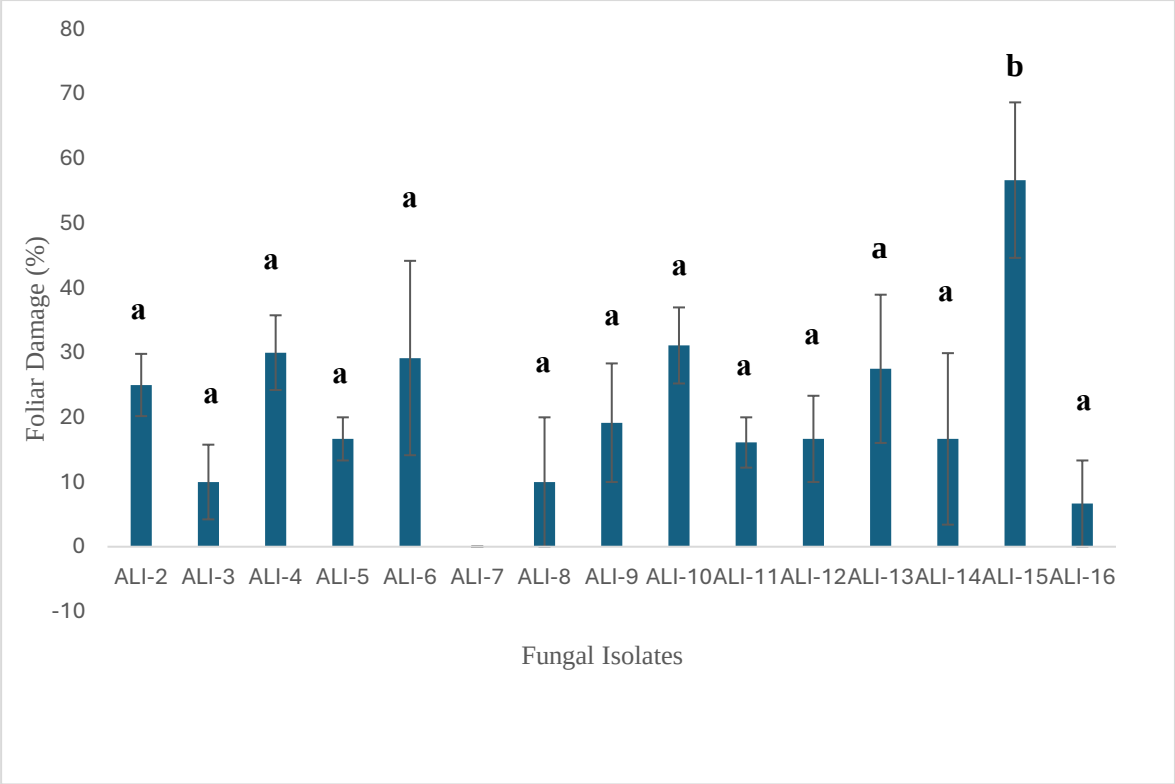


Fig.7.4.18. Effect of different fungal isolates on Alligator weed after 05 days of inoculation. Vertical bars represent the standard error of the mean. Means marked with the same letter(s) above the bars are not significantly different according to Bonferroni's test ($p < 0.05$).

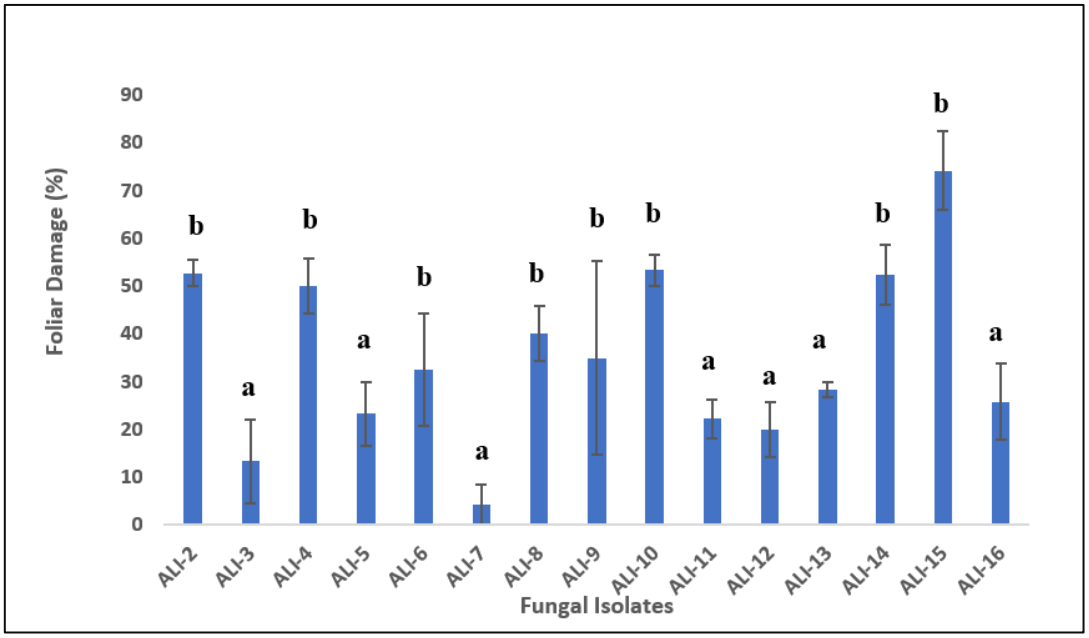


Fig.7.4.19. Effect of different fungal isolates on Alligator weed after 07 days of inoculation. Vertical bars represent the standard error of the mean. Means marked with the same letter(s) above the bars are not significantly different according to Bonferroni's test ($p < 0.05$).

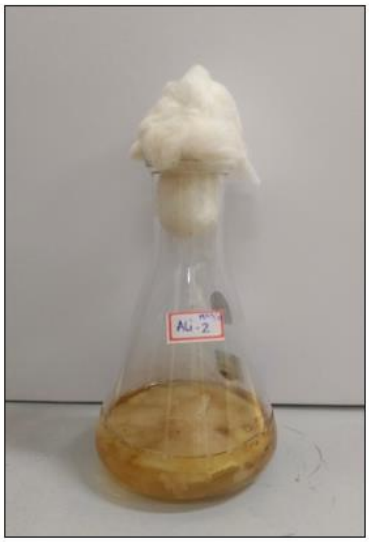
Discussions:

Among the fifteen fungal isolates, five isolates (ALI-2, ALI-4, ALI-10, ALI-14, ALI-15) caused leaf damage within 07 days of fungal inoculation. The other isolated exhibited relatively mild or no disease.

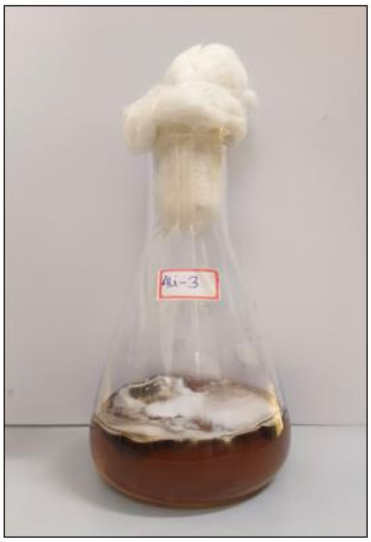
No disease or damage was observed in the control.

Five fungal isolates showing promising results in whole plant bioassay were assessed by toxicity assay of crude metabolites to test the bioherbicidal potential.

7.5. (A) Mass culture of fungus

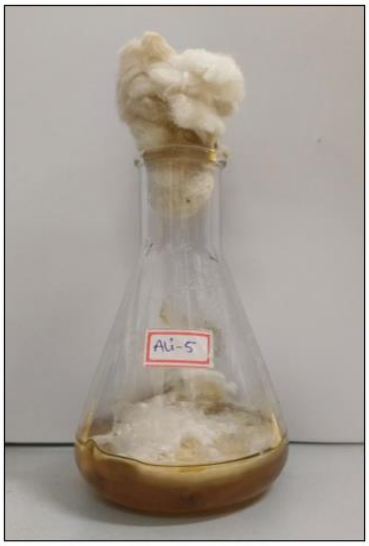


ALI-2

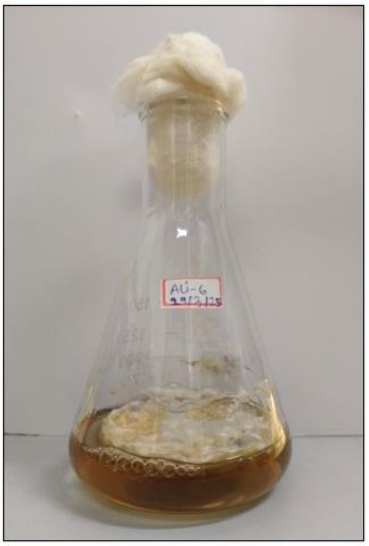


ALI-3

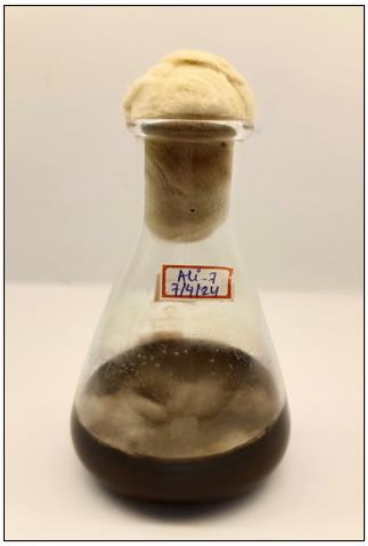
ALI-4



ALI-5

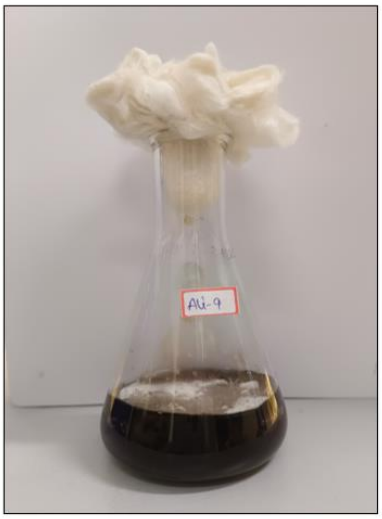


ALI-6



ALI-7

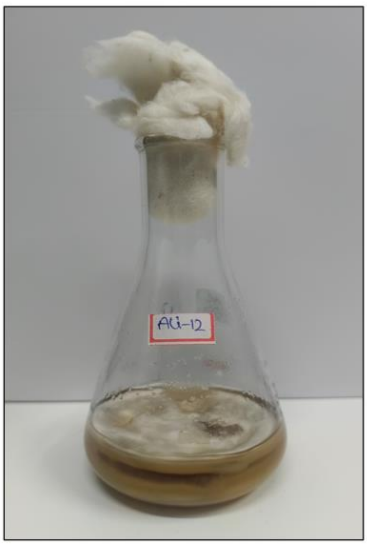
ALI-8



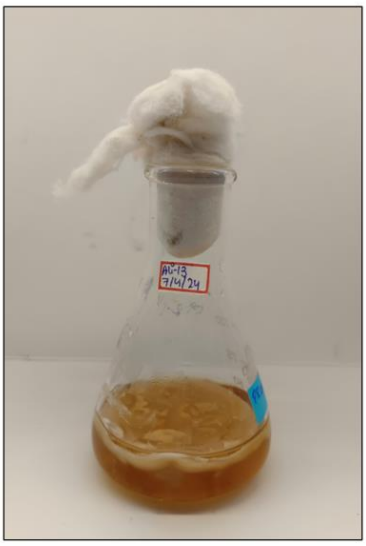
ALI-9

ALI-10

ALI-11



ALI-12



ALI-13

ALI-14

ALI-15

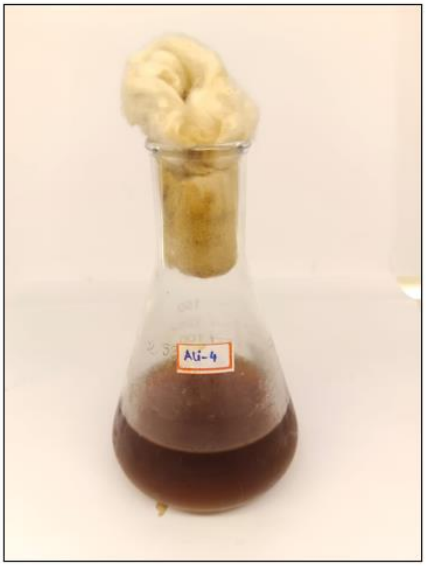
ALI-16

Fig.7.5.1. (A) Mass culture of all fungal isolates done to release metabolites, observed after 21 days of inoculation on PDB

7.5. (B) Extraction of Crude metabolites

Isolates that gave best results in whole plant bioassay were selected for extraction of crude metabolites

ALI-2



ALI-4

ALI-10

ALI-14

ALI-15

Fig.7.5.1. (B) Extracted cell-free crude metabolites of the selected fungal isolates which gave best results after whole plant bioassay

7.6. Toxicity test of extracted crude metabolites

Metabolites of five isolates (ALI-2, ALI-4, ALI-10, ALI-14, ALI-15) that demonstrated highest levels of phytotoxicity on whole plant assay of alligator weed were assessed in the metabolite assay.

(1) Control:

Healthy whole plant of Alligator weed + distilled water

DAY 05 **DAY 10** **DAY 15**

Fig 7.6.1. Toxicity test of crude metabolites: Control with distilled water as observed on day 05, day 10 and day 15

(2) ALI-2

Healthy whole plant of Alligator weed + metabolite extracted from ALI-2 fungus

DAY 05 **DAY 10** **DAY 15**

Fig 7.6.2. Toxicity test of crude metabolites: metabolite of ALI-2 as observed on day 05, day 10 and day 15

(3) ALI-4

Healthy whole plant of Alligator weed + metabolite extracted from ALI-4 fungus

DAY 05 **DAY 10** **DAY 15**

Fig 7.6.3. Toxicity test of crude metabolites: metabolite of ALI-4 as observed on day 05, day 10 and day 15

(4) ALI-10

Healthy whole plant of Alligator weed + metabolite extracted from ALI-10 fungus

DAY 05 **DAY 10** **DAY 15**

Fig 7.6.4. Toxicity test of crude metabolites: metabolite of ALI-10 as observed on day 05, day 10 and day 15

(5) ALI-14

Healthy whole plant of Alligator weed + metabolite extracted from ALI-14 fungus

DAY 05 **DAY 10** **DAY 15**

Fig 7.6.5. Toxicity test of crude metabolites: metabolite of ALI-14 as observed on day 05, day 10 and day 15

(6) ALI-15

Healthy whole plant of Alligator weed + metabolite extracted from ALI-15 fungus

DAY 05 **DAY 10** **DAY 15**

Fig 7.6.6. Toxicity test of crude metabolites: metabolite of ALI-15 as observed on day 05, day 10 and day 15

Results:

Fungal Isolates	FD (%) Mean ± SE
ALI-2	75.55 ± 4.44
ALI-4	38.88 ± 1.38
ALI-10	17.22 ± 4.33
ALI-14	13.61 ± 3.41
ALI-15	18.33 ± 4.40

Table 7.6.1. FD (%) Mean of Toxicity test of crude metabolites 15 days after crude metabolite spray

ANOVA Table

			Sum of Squares	df	Mean Square	F	Sig.
FD(%) * STRAIN	Between Groups	(Combined)	4780.370	4	1195.093	4.013	.034
	Within Groups		2978.241	10	297.824		
	Total		7758.611	14			

Table 7.6.2. ANOVA Table for Toxicity test of crude metabolites of the five fungal isolates after 15 days

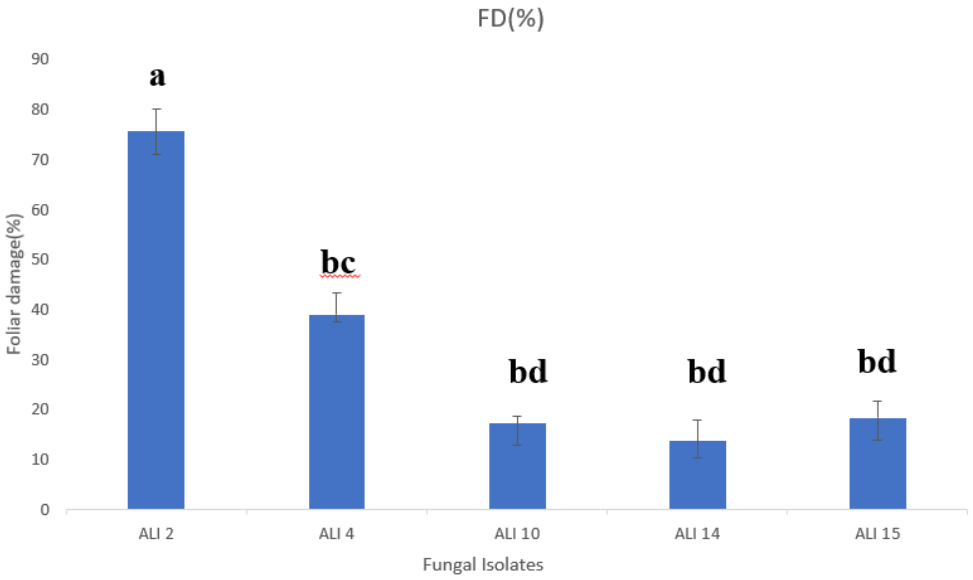


Fig.7.6.7. Effect of crude metabolites of different fungal isolates on alligator weed after 15 days of inoculation. Vertical bars represent the standard error of the mean. Means marked with the same letter(s) above the bars are not significantly different according to Bonferroni's test ($p < 0.05$).

Discussion: Five fungal isolates – ALI-2, ALI-4, ALI-10, ALI-14, and ALI-15, that exhibited strong phytopathogenic effects were selected for metabolite toxicity testing. Among these, only the crude metabolite derived from isolate ALI-2 demonstrated significant phytotoxicity when applied to alligator weed leaves. In contrast, the metabolites from the other four isolates exhibited mild toxic effects. When applied, metabolite of ALI-2 caused chlorosis of the leaves.

7.7. Morphological Identification

(1) ALI-2

Fig.7.7.1. Spores of fungal strain ALI-2 as observed under microscope at 40X magnification.

Discussion: Initially, ALI-2 is identified as *Alternaria* sp.

Alternaria sp. produced very few spores on Potato dextrose agar (PDA). However, large number of spores were observed on *Alternaria* sp. infected alligator weed leaves when observed under microscope at 40X magnification.

CHAPTER 08

SIGNIFICANCE OF THE STUDY

The biological management of alligator weed (*A. philoxeroides*), found in both aquatic and terrestrial environments, is crucial for ecological, agricultural, and environmental health. This research seeks to search and assess the potential biocontrol agents, especially phytopathogenic fungi, that could be developed into effective bioherbicides. Investigating and utilizing fungal pathogens and their metabolites as control methods presents a sustainable and eco-friendly alternative to chemical herbicides, which often threaten non-target species and contribute to environmental degradation.

Alligator weed is well-known for its rapid proliferation and its capacity to outcompete native plant species, resulting in a loss of biodiversity, blockage of waterways, and degradation of agricultural land (Julien et al., 1995). Traditional approaches like mechanical removal and chemical herbicides only offer temporary solutions and frequently cannot stop regrowth. Additionally, the overuse of chemical herbicides may lead to herbicide resistance, deterioration of soil quality, and pollution of aquatic ecosystems (Duke et al., 2012). In contrast, utilizing fungal bioherbicides for biological control provides a precise method that can manage weed populations while preserving beneficial plant species and the larger ecosystem. Fungal pathogens generate various secondary metabolites—like enzymes, toxins, and growth inhibitors—that can interfere with essential physiological functions in the weed, ultimately leading to its decline (Charudattan et al., 2001). These bioherbicides are biodegradable, selective, and likely less harmful to both human health and the environment.

By isolating fungi that are either native or specifically pathogenic to alligator weed, this study aids in the creation of mycoherbicides suitable for use in field environments. Incorporating these biological control agents into weed management strategies supports the concepts of integrated pest management, which promotes minimizing chemical usage and enhancing dependence on biological and ecological mechanisms (Boyetchko et al., 2009). This study aims at identifying highly pathogenic fungal isolate with strong potential as a biocontrol agent against alligator weed. Among the screened fungi, the most effective strains that exhibits rapid infection, tissue necrosis, and high sporulation, makes it suitable for further development into a mycoherbicide.

The identification of this fungus provides a strong foundation for future research into bioherbicidal formulation, mode of action studies, and field-level application for eco-friendly weed control. By providing these contributions, the study tackles an important issue related to invasive species while also aiding worldwide initiatives aimed at sustainable agriculture and the preservation of biodiversity.

CHAPTER 09

FUTURE PROSPECTS

To advance the development of the identified fungal isolate as a reliable and environmentally safe bioherbicide, several critical research directions must be pursued. First and foremost, comprehensive host range testing is essential. To determine the host range of a phytopathogenic fungus experimental inoculation is conducted. This involves evaluating the impact of the fungus on a wide variety of plant species, including native flora and economically significant crops where they are observed over time for disease symptoms like wilting, necrosis or chlorosis. Ensuring the specificity of the biocontrol agent is crucial to avoid unintended ecological consequences, such as damage to non-target plants or disruption of local biodiversity. Only strains with a narrow host range, specifically targeting *A. philoxeroides*, should be considered for further development.

In addition, molecular identification and characterization of the fungal strain should be carried out using advanced techniques such as DNA sequencing (Basak, Dutta and Ray, 2024). Accurate identity of the organism confirms the insights of its genetic potential, pathogenic mechanisms, and safety profile. Molecular tools can also be used to assess genetic stability and differentiate the strain from closely related, potentially harmful species.

Furthermore, future research should focus on optimizing the formulation and delivery methods of both the fungal spores and their bioactive metabolites to enhance efficacy under diverse environmental conditions. Long-term field trials will be vital to evaluate the consistency of weed suppression, persistence of the agent in natural ecosystems, and overall environmental impact.

These steps are essential to ensure the scientific validity, regulatory acceptance, and commercial viability of the fungus as a bioherbicidal product. Through rigorous testing and characterization, this study can contribute to the broader goals of sustainable weed management and reduced reliance on chemical herbicides.

CHAPTER 10

CONCLUSION

As per the study, five fungal isolates ALI-2, ALI-4, ALI-10, ALI-14 and ALI-15 has been shown to produce disease symptoms in *A. philoxeroides*. Out of these five isolates, crude metabolite of ALI-2 is observed to exert the most phytotoxic effect on Alligator weed. The crude metabolite derived from the isolate demonstrated significant phytotoxicity when applied to alligator weed leaves. When applied, crude metabolite of *Alternaria* sp. caused chlorosis of the leaves of healthy alligator weed.

After primary morphological identification, it is identified as *Alternaria* sp.

It is reported to cause severe disease on *A. philoxeroides* in many countries, including India. Hence, it can be concluded that this species is distributed around the world and possess a bioherbicidal capability against alligator weed and utilised in biocontrol studies.

CHAPTER 11

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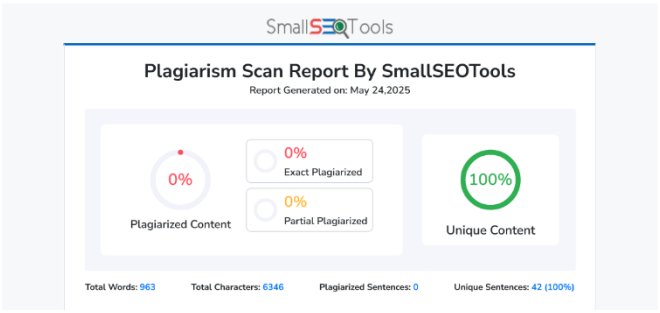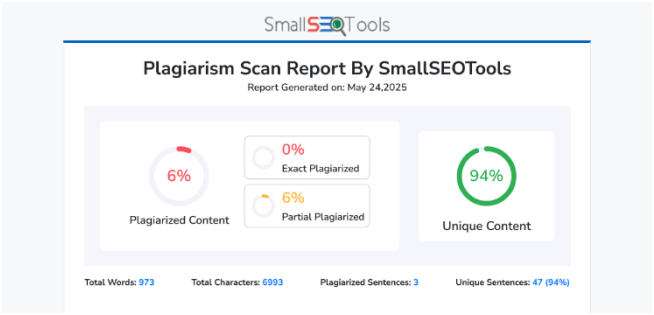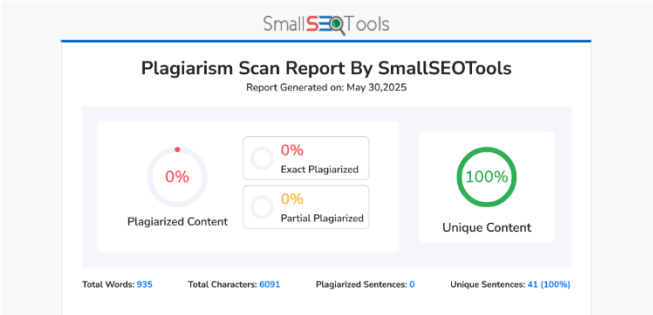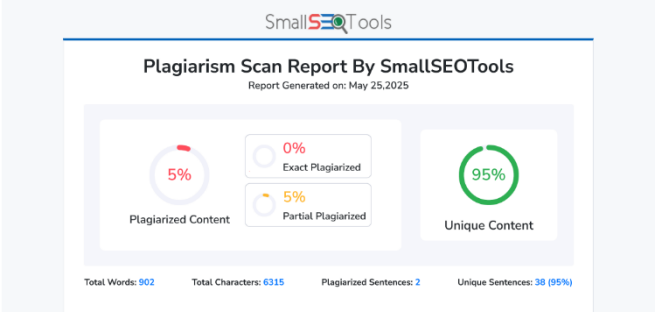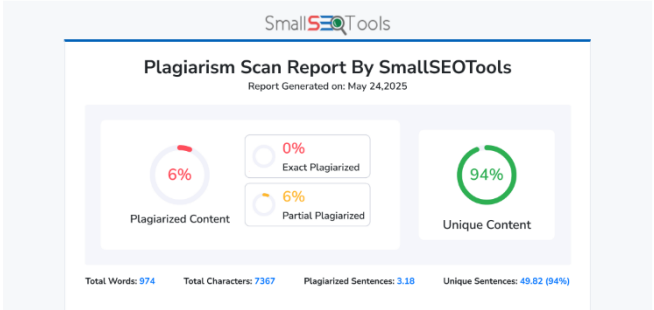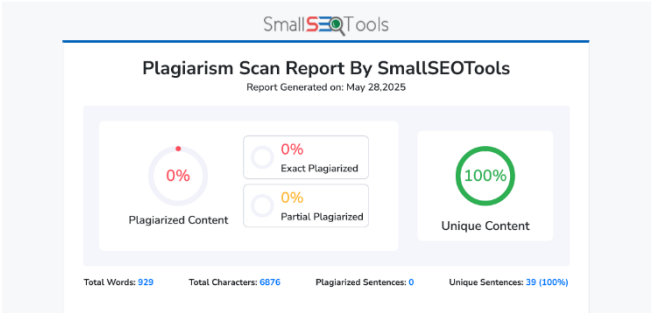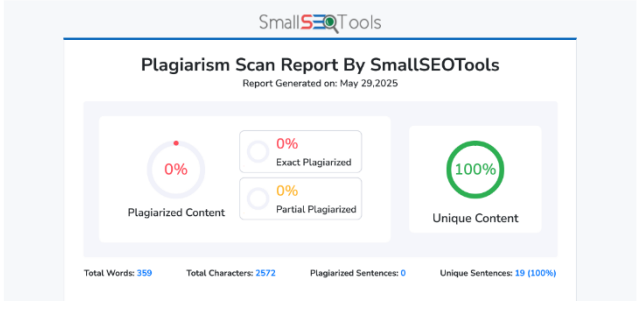
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